MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *Escherichia coli* IN CAPTIVE AND WILD OLIVE BABOON (*Papio anubis*) GUT

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Molecular Characterization and Antimicrobial Susceptibility Patterns of *Escherichia coli* in Captive and Wild Olive Baboon (*Papio anubis*) Gut

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

I dedicate this project to God Almighty who gave me the strength and courage to keep moving in spite of darkness and numerous distractions.

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ABBREVIATIONS AND ACRONYMS

AA:	Aggregative adherence
AAALAC:	Association for Assessment and Accreditation of Laboratory Animal Care
AAF:	Aggregative Adherence Fimbriae
AIDS:	Acquired Immunodeficiency Syndrome
AMR:	Antimicrobial resistance
ABC:	ATP binding cassette
bp:	Base pairs
bfp:	Bundle forming pilus
cdt:	Cytolethal distending toxin
CF:	Colonization Factor
CFA:	Colonization Factor Antigens
CLSI:	Clinical Laboratory Standards Institute
CNF:	Cytotoxic Necrotizing Factors
DEC:	Diarrheagenic *E. coli*
DAEC:	Diffuse aggregative *E. coli*
DNA:	Deoxyribonucleic acid
EAEC:	Enteroaggregative *E. coli*
EHEC:	Enterohaemorrhagic *E. coli*
EIA:	Enzyme Immunoassay

EIEC: Enteroinvasive *E. coli*

ELISA: Enzyme-linked immunosorbent assay

EPEC: Enteropathogenic *E. coli*

ER: Endoplasmic reticulum

ESBL: Extended Spectrum Beta Lactamase

ETEC: Enterotoxigenic *E. coli*

FAS: Fluorescence Actin Staining

FITC: Fluorescin Isothiocyanate

GIT: Gastrointestinal Tract

HC: Hemorrhagic Colitis

IRC: IPR's Institutional Review Committee

LEE: Locus of Enterocyte Effacement

LT: Labile Toxin

MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

MDR: Multi-drug resistant

NHP: Non-Human Primate

NM: Non Motile

PCR: Polymerase Chain Reaction

qPCR: Quantitative PCR

ST: Stable Toxin

STEC: Shiga-toxigenic *E. coli*

ABSTRACT

Escherichia coli are normal microflora in the gut of warm-blooded animals but some strains are pathogenic to humans causing fatal diarrhea. Cases of antimicrobial resistance have been reported on *E. coli* isolated from different domestic and wild animals indicating that they are potential reservoirs for zoonotic transmission of both pathogenic and resistant strains of bacteria. Zoonotic transmission is favoured by anthropogenic activities. This study was aimed at undertaking comparative characterization of *E. coli* pathotypes that colonize the gut of captive and wild olive baboons (*Papio anubis*) as well as determining antimicrobial susceptibility profiles and presence of extended spectrum β-lactamase genes in all *E. coli* isolates. Stool samples were collected from a total of 124 olive baboons divided into two equal groups of captive and wild. All *E. coli* were isolated by culture-based technique and characterized using polymerase chain reaction to determine of virulence genes associated with each pathotype. All *E. coli* isolates were subjected to susceptibility testing to fourteen antimicrobial agents followed by characterization of three putative resistance genes; $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. Enteropathogenic, enterotoxigenic, enteroinvasive and enterohaemorrhagic *E. coli* were detected from both captive and wild baboons. However, enteropathogenic, enterotoxigenic and enteroinvasive isolates were detected in 29.0% of wild baboons whereas carriage of enterotoxigenic, enteropathogenic, enteroinvasive and enterohaemorrhagic isolates occurred in 24.2% of captive population ($p<0.05$). Wild olive baboons appeared to harbor more enteropathogenic *E. coli* (22.6%) compared to the captive population (4.8%). On the other hand, prevalence of enterotoxigenic *E. coli* was higher among the captive olive baboons (14.5%) compared to the wild population (1.6%). *E. coli* isolates from both groups of animals were resistant to all antimicrobial agents except ciprofloxacin. Prevalence of ampicillin resistance was high in *E. coli* isolated from both wild (35.5%) and captive (32.3%) baboons. There was higher prevalence of Extended Spectrum β-Lactamases in *E. coli* isolated from wild (17.7%) than captive (14.5%) baboons ($p<0.05$). Carriage of bla_{SHV} gene was higher among *E. coli* isolates from wild olive baboons (11.3%) compared to the captive population (1.6%). There was higher prevalence of $bla_{\text{CTX-M}}$ (8.1%) and bla_{TEM} (4.8%) in captive olive

baboons compared to the wild population (3.2%) although these differences were not significant ($p>0.05$). This study demonstrates that the gut of both captive and wild populations of olive baboons is colonized by *E. coli* that are not only pathogenic to humans but also harbor extended-spectrum β-lactamases that are highly transmissible. As reservoirs of *E. coli* pathotypes and extended spectrum β-lactamases producers, baboons could play a potential role not only in transmission of diarrheal diseases, but also of antibiotic resistance genes to the environment and other animals including humans. There is need for further investigations to characterize additional antimicrobial resistance genes and their variants. Regular training of laboratory animal care staff on prevention and control of zoonotic transmission of diarrheagenic *E. coli* is required.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Diarrhoeal diseases can be caused by microbes that range from bacteria, protozoa to viruses and manifest as watery, secretory, bloody diarrhea or gastroenteritis (Gonzales-Siles & Sjöling, 2016). Over 2.5 billion cases of diarrhoeal infections and 760,000 diarrhoea associated deaths are reported annually among children aged below five years making it the second leading cause of morbidity and mortality after pneumonia (Liu *et al.*, 2012). It has also been shown that diarrhea can lead to malnutrition, immune deficiencies and other long-term effects including stunting and cognitive impairment in newborn and toddlers thus making it a serious health problem (Gonzales-Siles & Sjöling, 2016; Niehaus *et al*., 2002). Several studies have proven that non-human primates (NHPs) are useful models for pre-clinical studies on numerous conditions of public health importance (Chai *et al.*, 2007; Kagira *et al*., 2011; Valdés *et al*., 2013). They are also known to harbor zoonotic pathogens including but not limited to *E. coli* which are commensals in the gut of most warm blooded animals where they prevent colonization by other bacteria that may be pathogenic (Bailey & Mansfield, 2010). Some strains of *E. coli* are pathogenic (Clayton *et al.*, 2014) causing diverse gastrointestinal diseases in humans ranging from mild diarrhoea to severe diseases such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS) especially among children and the elderly (Nguyen & Sperandio, 2012). The main diarrhegenic *E. coli* (DEC) include: enterotoxigenic *E. coli* (ETEC) that causes diarrhea by heat labile and/or heat stable toxins, enteropathogenic *E. coli* (EPEC) which is associated with profuse diarrhea, enteroinvasive *E. coli* (EIEC) which is associated with watery to bloody diarrhea as it invades the colon epithelial lining, enteroaggregative *E. coli* (EAEC) that causes persistent diarrhea in neonates and children (Jafari *et al.*, 2012) while enterohaemorrhagic *E coli* (EHEC) particularly O157:H7 strains are associated with bloody diarrhea which may progress to a serious complications, the HUS (Regua-Mangia *et al.*, 2012). Diarrhea in captive primates is an age long problem in both zoo settings and

research colonies. One of the most pathogenic forms of *E. coli* in humans; Shiga toxin-producing *E. coli* (STEC), which includes serotype 0157:H7. STEC are commensal organisms in ruminants, but are highly virulent pathogens in humans and may cause severe hemorrhagic colitis (Ferens & Hovde, 2011). In NHPs, it is unclear whether certain pathotypes exist as commensals or pathogens (Clayton *et al.*, 2014) but their presence constitute a potential source of zoonotic diarrhoeal infections to humans.

Globally, there is a growing concern over antimicrobial resistance (AMR) which has translated into high morbidity and mortality in both humans and animals (Wallensten *et al*., 2011). Over the past decade there has been a rapid increase in development of AMR by previously susceptible bacteria against different agents by varied mechanisms (Szmolka & Nagy, 2013). Large amounts of antibiotics used for therapy have resulted in the selection of pathogenic bacteria resistant to multiple drugs. Complications that arise from antibiotics resistant pathogens increases the severity of the infections that require sophisticated management including prolonged chemotherapy and even hospitalization (Alanis, 2005; Kiiru *et al*., 2012; Okeke *et al*., 2007).

Management of bacterial diarrhoea is compounded by excessive use of antimicrobial agents that has exerted selective pressure on bacteria to acquire resistance mechanisms against them. Extended spectrum β-lactamases (ESBLs) can hydrolyze third generation cephalosporins and aztreonam but are inhibited by clavulanic acid and other β-lactamase inhibitors (Paterson & Bonomo, 2005). Genes encoding for ESBLs are located on plasmids borne by members of the family enterobacteriaceae including *E. coli* implying that there is a high risk of plasmid-mediated transfer of resistance across bacterial species (Isendahl *et al*., 2012). Such availability of additional potential source of DEC infections coupled with antimicrobial resistance can result in high morbidity and mortality among the infected human hosts (Wallensten *et al.*, 2011).

1.2 Problem statement

The gut of olive baboons (*P. anubis*) is normally colonized by *E. coli* as commensals (Kolappaswamy *et al.*, 2014; Lugano *et al*,.2018). However, some strains of this bacterium

are known to be serious pathogens to humans (Heidary *et al*., 2014) capable of causing zoonotic infections that range from mild diarrhoea to devastating severe infections with fatal complications (Bailey & Mansfield, 2010). Transmission to humans is favoured by their close interactions with olive baboons due to forest encroachments as its cover reduces, bush meat eating and sharing of water sources (Razzak *et al.*, 2015; Rwego *et al*., 2008). Urbanization has also brought these NHPs closer to humans; a move with potential to increase the risk of zoonoses including diarrhoeal diseases caused by *E. coli* arising from contact with fecal material from infected olive baboons (Hahn *et al*., 2003). This translates to high morbidity due to DEC infections and mortality in the high risk groups; children and the elderly. Management of infections caused by these bacteria is complicated by the rapidly developing problem of AMR with an upsurge in cases of multi drug resistant (MDR) *E. coli* being reported globally (Maragakis & Perl, 2010). Zoonotic transmission of antibiotic resistant bacteria including *E. coli* is not uncommon and the situation is aggravated by the fact that genes encoding this resistance can be transferred horizontally across microbes through mobile genetic elements and conjugative plasmids (Hunter *et al*., 2010; Iyer *et al*., 2013).

1.3 Justification

Diarrhegenic *E. coli* constitute infectious zoonoses that range from gastroenteritis to serious devastating infections to humans, with the elderly, infants and children being the most susceptible. Limited studies conducted on NHPs have not clearly demonstrated whether *E. coli* pathotypes are merely commensals or pathogens except in rhesus and cynomolgus macaques where fatal diarrhea outbreak was attributed to EIEC (Clayton *et al.*, 2014; Kolappaswamy *et al.*, 2014). Previous studies on Amboseli National park baboon troops only focused on antimicrobial susceptibility patterns of *E. coli* leaving out ESBLs and *E. coli* pathotypes (Rolland *et al*.,1985). Mureithi *et al*., (2015) focused on antimicrobial resistance of *E. coli* isolated from olive baboons that have not been previously exposed to antibiotics but did not characterize the pathotypes. Humans share habitats like water sources which increase the potential for transmission of DEC. Characterization DEC isolated from olive baboons will therefore be useful in contributing

towards prevention of the disease through understanding of additional potential source/reservoir of infection by virtue of human interactions with these NHPs. The ultimate output from this study will translate into reduction in morbidity and mortality among children who constitute the high risk group.

1.4 Research questions

1. Which pathotypes of *E. coli* colonize the gut of captive and wild olive baboon (*Papio anubis*)?
2. What is the antimicrobial susceptibility profile of *E. coli* isolated from captive and wild olive baboon (*Papio anubis*) gut?
3. Which extended spectrum β-lactamase-encoding genes are carried by *E. coli* isolated from captive and wild olive baboon (*Papio anubis*) gut?

1.5 Objectives

General objective

To determine the prevalence of *E. coli* pathotypes and antimicrobial resistance genes among *E. coli* isolated from captive and wild olive baboon (*Papio anubis*) gut.

Specific objectives

1. To determine pathotypes of *E. coli* isolated from captive and wild olive baboon (*Papio anubis*) gut.
2. To determine antimicrobial susceptibility patterns of *E. coli* isolated from captive and wild olive baboon (*Papio anubis*) gut.
3. To determine presence of selected extended spectrum β-lactamase-encoding genes found in *E. coli* isolated from captive and wild olive baboon (*Papio anubis*) gut.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of diarrheagenic *Escherichia coli*

Diarrhegenic *E. coli* belong to the family enterobacteriaceae and the tribe Escherichia which constitute a group of motile Gram negative rods. Pathogenic *E. coli* colonize mucosal surfaces, evade host defense, multiply and cause damage to the host. These pathogens have unique ability to invade and adhere to intestinal mucosal sites despite peristalsis and microbial antagonism. Generally, all *E. coli* possess surface adherence fimbria but DEC strains form specific fimbrial antigens that enhance intestinal colonization as well as allowing adherence to the mucosa of small intestines (Kaper *et al.*, 2004). The pathogenetic mechanisms employed by DEC include: production of enterotoxins by ETEC and EAEC, invasion by EIEC, intimate adherence coupled with membrane signaling by EPEC and EHEC (Nataro & Kaper, 1998).

2.2 Enterotoxigenic *Escherichia coli* infections

Enterotoxigentic *E. coli is* known to cause diarrhea among children in developing countries and in travelers (Bölin et al., 2006). This pathotype is most common in areas of low income settings with inability to afford proper hygiene and safe drinking water where it is estimated to cause 2.5 million cases of infection with 700,000 children under five years succumbing to the infection (Kotloff *et al.*, 2013; Lamberti *et al*., 2014). Infections by ETEC have also been associated with food borne illnesses (MacDonald *et al*., 2015; Pakalniskiene *et al*., 2009). The microbe expresses plasmid-borne enterotoxins; the heat labile toxin (LT) and/or the heat stable toxin (ST) that both mediate deregulation of membrane ion channels in the epithelial membrane (Fleckenstein *et al*., 2010). Each ETEC isolate typically expresses one to three colonization factor antigens (CFAs) that mediate adhesion to the epithelium of which CFA/I and coli surface antigens 1–6 (CS1-CS6) are most prevalent although CS7, CS14 and CS17 are also common (Gonzales-Siles

& Sjöling, 2016; Madhavan & Sakellaris, 2015). Watery diarrhoea caused by ETEC poses a direct risk of transmission by shared toilets or through water transmission. Presence of ETEC in rivers, drinking water and in irrigation water has been demonstrated (Ahmed *et al*., 2013; Begum *et al*., 2007) and ETEC is able to adhere firmly to fresh vegetables, which increases risk for transmission (Shaw *et al*., 2011). Strains of ETEC are therefore present in the environment and must adapt to and survive, harsh conditions. On the other hand, the human gastrointestinal tract (GIT) is an equally hostile environment and any successful pathogen needs to be able to utilize nutrients available in the gut and also to sense the environment for proper expression of virulence factors (Gonzales-Siles & Sjöling, 2016).

2.2.1 Diagnosis of Enterotoxigenic *Escherichia coli* infections

This strain produces enterotoxins; labile toxin (LT) and/or stable toxin (ST) which form the basis of their identification (Fleckenstein *et al*., 2010). As the gold standard for identification of LT and ST, the rabbit ileal loop model and the infant mouse assay have been used respectively (Gomes *et al*., 2016). LT is strongly immunogenic unlike ST and can therefore be detected by its direct action on two tissue culture cell lines, Y1 adrenal cells and Chinese hamster ovarian cells where they produce physiological changes specific for LT and can be neutralized by antitoxin (Gomes *et al*., 2016). The two tissue culture assays have widely been used for LT recognition until the development of the enzyme-linked immunosorbent assay (ELISA)which was later developed to detect both LT and ST (Qadri *et al*., 2005). Other specific assays including staphylococcal coagglutination, passive latex agglutination, immunoprecipitation in agar, and the Biken test are available, though they have not been used for diagnosis (Alam & Ashraf, 2003). Current diagnosis is based on detection of LT and ST directly on fecal material as well as isolated colonies using molecular based techniques; conventional PCR and quantitative real-time PCR (Galbadage *et al*., 2009).

2.3 Enteropathogenic *Escherichia coli* infections

Enteropathogenic *E. coli* were originally associated with infantile diarrhea but later it was observed that they were over-diagnosed. This led to their definition by their characteristic localized adherence pattern in tissue cultured cells which finally settled on use of specific virulence genes as the current basis of their identification (Ochoa & Contreras, 2011). Strains of EPEC possess the ability to produce attaching and effacing (A/E) lesions which allow the bacteria attach tightly to the host cell membrane causing a disruption of the cell surface leading to effacement of microvilli (Gomes *et al*., 2016). Intestinal cell attachment is mediated by an outer membrane protein called intimin, encoded by *eae*, which is currently used for the molecular diagnosis of EPEC (Hernandes *et al*., 2009; Trabulsi *et al*, 2002). Genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island that contains the genes encoding intimin, a type III secretion system, a number of secreted enteroccocal surface proteins (Esp), and the translocated intimin receptor named Tir. Two LEE insertion sites have been described on the *E. coli* chromosome, and a third unidentified insertion site has been reported (Trabulsi *et al*., 2002). Based on molecular characterization, EPEC are classified into typical and atypical strains based on the presence of the plasmid *E. coli* adherence factor (EAF), bundle forming pilus *(bfp*) and plasmid encoded regulator(*per*). These two encode type IV *bfp* and a transcriptional activator *per* respectively. All EPEC lack genes to produce (*stx*) implying that typical strains are *eae*+*bfp*A+*stx*− and produce the localized adherence (LA) phenotype associated with the production of *bfp*. *E. coli* strains that are *eae*+*bfp*A−*stx*− are classified as atypical EPEC (aEPEC) and they display localized-like (LAL), diffuse adherence (DA), or aggregative adherence (AA) patterns (Ochoa & Contreras, 2011). The LAL pattern in aEPEC is associated with the *E. coli* common pilus and other known adhesins (Scaletsky *et al.*, 2010).

2.3.1 Diagnosis of Enteropathogenic *Escherichia coli* infections

Diagnosis of EPEC is made based on pathogenic characteristics that distinguish it from other *E. coli* species and subdivide EPEC into “typical” and “atypical” categories. EPEC

is defined based on phenotypic properties, such as LA and A/E histopathology, that can be readily assessed using microscopy and cell culture techniques, as well as by the presence or absence of genetic elements such as *eae*, *bfp*, and *stx* (Nataro & Kaper, 1998). Patches of filamentous actin beneath A/E bacteria on the surface of cultured epithelial cells can be demonstrated by fluorescin actin staining using fluorescin isothiocyanate (FITC) or rhodamine-conjugate phalloidin. tEPEC can be distinguished from aEPEC and other DEC like DAEC and EAEC by HeLa or Hep-2 cell adherence assay (Hernandes *et al*., 2009). Due to lack of tissue culture facilities in most clinical laboratories to perform Hep cell adherence or fluorescence actin staining (FAS) assays detection is routinely done by deoxyribonucleic acid (DNA) probe hybridization or Polymerase chain reaction (PCR) based screens targeting *eae, bfp,* and EAF sequences (Croxen *et al*., 2013).

2.4 Enteroinvasive *Escherichia coli* infections

Enteroinvasive *E. coli* strains are biochemically, genetically, and pathogenetically related to *Shigella* species with a proposal to reclassify shigellae as one species in genus *Escherichia* (Lan *et al.*, 2004; Peng *et al.*, 2009). Strains of EIEC possess invasive plasmid (pINV) encoding the ability to invade host tissues infect the colonic mucosa by invading M cells, macrophages and epithelial cells resulting in a watery diarrhea, which in severe cases may be followed by the onset of scanty dysenteric stools containing blood and mucus (Jafari *et al*., 2012). A plasmid encodes insertion sequence (IS) elements and contains a 30 kb region enabling the bacteria to invade intestinal epithelial cells (Parsot, 2005). Components of type three secretion system (T3SS) such as translocators, transcriptional activators, some effectors and chaperones are coded by this region with the expression of the invasive (*inv*) encoded genes being regulated globally by VirB and MxiE (Johnson & Nolan, 2009).

2.4.1 Diagnosis of Enteroinvasive *Escherichia coli* infections

Enteroinvasive *E. coli* causes production of stools that contain blood and pus. Invasiveness of EIEC in stool samples can be detected by Sereny test that demonstrates the ability of

the toxin to cause keratoconjuctivitis in guinea pigs (Kopecko, 1994). Culture-based techniques can also be used to isolate and identify EIEC. Stool is cultured onto differential/selective culture media then identified by inability to ferment lactose and utilize citrate; absence of motility, lysine decarboxylase, and urease activity; and acid but no gas and H_2S production upon sugar fermentation (Niyogi, 2005; Panchalingam *et al*., 2012). PCR can also be used to detect pathotype-specific genetic markers, like the invasion plasmid antigen H gene (*ipaH*) or the invasion-associated locus gene (*ial*) ((Mohammadzadeh *et al.*, 1989). TaqMan Array Card platform that can multiplex up to 384 targets and has been used to detect 19 pathogens, including EIEC, is a prospective tool for fast and comprehensive surveillance data processing on the next level of multiplexing technologies (Liu *et al*., 2013).

2.5 Enteroaggregative *Escherichia coli* infections

This pathotype is the most recently identified DEC and is the second most common cause of travelers' diarrhea after ETEC in both developed and developing countries (Jafari *et al*., 2012). Globally, EAEC have been associated with endemic and epidemic diarrhea besides their recent demonstration as cause of acute diarrheal illness in newborns and children in industrialized countries. This organism has also been associated with persistent watery diarrhea which may be accompanied by mucus or blood (Croxen & Finlay, 2010; Harrington *et al.*, 2006). Discovery of EAEC as well as diffusely adherent *E. coli* (DAEC) stemmed from the studies showing that EPEC adhere to HEp-2 cells in a distinctive pattern. Examination of a collection of DEC strains that are not of EPEC serogroups have shown that many of these strains also adhere to HEp-2 cells and the phenotype is different from that of EPEC. This pattern of adherence, which had been called "diffuse" was subsequently subdivided into aggregative and true diffuse adherence. Aggregative adhesion (AA) is the hallmark of EAEC that involve formation of a stacked brick pattern on HEp-2 cells besides their auto agglutination (Jafari *et al*., 2012).

Pathogenesis of EAEC is characterized by colonization of intestinal mucosa, mucoid biofilm formation and elaboration of various enterotoxins, cytotoxins and mucosal

inflammation (Croxen & Finlay, 2010; Harrington *et al*., 2006). Colonization of intestinal mucosa occurs via aggregative adherence fimbriae (AAF/I-IV) encoded by a plasmid; pAA (Boisen *et al.*, 2008). Adhesion of EAEC to intestinal tissue is mediated by antigenically heterogeneous adhesins similar to those found in ETEC but multiple carriage of AAFs by an EAEC strain has been rare (Aslani *et al.*, 2011). Biogenesis of AAFs is regulated by transcriptional activator; *Agg*R which is encoded by pAAs and is also the major EAEC virulence regulator controlling diverse virulence genes encoded by pAAs as well as by chromosomes (Harrington *et al*., 2006). Adherence of EAEC to the mucosa is characterized by the formation of a thick, aggregating mucus layer inside which they survive and this biofilm production has been attributed to the activity of *fis* and *yafK* genes (Sheikh *et al.*, 2001). Movement of bacteria across cell surfaces for subsequent aggregation and adherence is facilitated by anti-aggregation protein (Aap) or dispersin which is highly immunogenic and is translocated via an ATP binding cassette (ABC) transporter complex (the aat apparatus). Both these genes have been used for identification and classification of EAEC isolates, but it has been noted that dispersin gene (*aap*) can be detected in DAEC as well as nonpathogenic *E.coli* (Monteiro *et al*., 2009).

2.5.1 Diagnosis of Enteroaggregative *Escherichia coli* infections

Isolated putative EAEC can be identified by sub-culturing them into Luria broth at 37^{o}C followed by infection of semi confluent Hep-2 cells for three hours to demonstrate pathognomic aggregates; the hallmark "stacked-brick" appearance, where the bacilli are elongated and sometimes line up in a single layer on the surface of the cell (Dudley *et al*., 2006). Molecular based detection of EAEC targets *agg*R gene (Huang *et al*., 2007).

2.6 Diffusely adherent *Escherichia coli* infections

Diffusely adherent *E. coli* strains form DA pattern on cultured epithelial HEp-2 as well as HeLa cells as a heterogeneous group that were previously subdivided into two subclasses: DAEC expressing Afa/Dr adhesins (Afa/Dr DAEC) and DAEC not expressing Afa/Dr adhesins (Servin, 2005). The subclass of DAEC that does not express Afa/Dr adhesins has

recently evolved with the main member of this subclass; the diarrhea-associated DAEC expressing the *aid*A gene, encoding an adhesin involved in diffuse adherence (AIDA-I), belonging to the newly defined second class of EPEC designated atypical EPEC (aEPEC) since it is *eae* positive (Benz & Schmidt, 1989; Servin, 2014). It has been shown that the relative risk of diarrhea associated with DAEC increases with age of children from 18 months to 5 years. The intestinal carriage of these strains has also been reported to be widespread in older children and adults. The consequences of this persistence are unknown, but several observations have suggested a potential role in the development of chronic inflammatory intestinal disease (Le Bouguénec & Servin, 2006).

2.6.1 Diagnosis of Diffusely adherent *Escherichia coli* infections

Adhesion assay is used to detect DAEC based on mannose resistant diffuse aggregation on cultured HEp-2 or HeLa cells where they form patterns that can be classified as localized, diffuse or aggregate but this method is not specific for Afa/Dr DAEC since other pathogenic *E. coli* strains may show similar patterns (Servin, 2005). Diffuse clustering assay (DCA) is used to detect HeLa cell receptors on Afa/Dr DAEC (Goluszko *et al*., 2001). Colony hybridization assays employing DNA probes like daaC which is a 30bp Pst1 fragment of a plasmid pSS1 has been widely used for detection of Afa/Dr DAEC (Snelling *et al*., 2009). PCR assays that amplify *afa* gene fragments have proved to be more promising since they are highly specific (Le Bouguénec *et al*., 2001; Servin, 2014).

2.7 Enterohaemorrhagic *Escherichia coli* infections

Enterohaemorrhagic *E. coli* is a subset of the Shiga-toxigenic *E. coli* (STEC) group of pathogens which cause disease that ranges from mild watery diarrhea to hemorrhagic colitis (HC) and in extreme scenarios hemolytic uremic syndrome (HUS). Infection by STEC is considered to be the most common foodborne zoonotic pathogen causing various disease conditions in both animals and humans with ruminants especially cattle as the most important source of infection primary reservoir (Perera *et al.*, 2015). In humans,

STEC infections may primarily result from consumption of undercooked beef, raw milk, meat and dairy products, vegetables, unpasteurized fruit juices, and water contaminated with fecal material from infected persons or animals (Neher *et al*., 2016). The virulent strains of STEC are associated with one or more types of *stx* (*stx*1, *stx*2 or *stx*2 variants) as well as the property of producing intimin, which is required for attachment effacement lesions encoded by *eae* gene (Blanco *et al*., 2004; Perera *et al*., 2015). There are, at least, 200 serotypes of *E. coli* that are capable of producing *sxts* but *E. coli* O157:H7 is the most well-known (Neher *et al*., 2016). Infections caused by EHEC O157:H7 have been associated with contaminated meat products but cases also appear in relation to contaminated fresh produce. Ruminants, most notably cattle, are the primary host for EHEC O157:H7, which they colonize asymptomatically at the recto-anal junction using the LEE-encoded T3SS. Virulence factors in EHEC O157:H7 includes a T3SS and its set of associated effector proteins, the *stx* toxin and the pO157 plasmid (Croxen & Finlay, 2010; Croxen *et al*., 2013). Variety of serotypes and virulence factors found among STEC strains, are responsible for varying severity of disease. *stx*1-containing STEC can lead to HUS while the presence of *stx*2 is associated with more severe human disease than that of *stx*1. Ingested STEC are able to survive low pH of the stomach (Hong *et al.*, 2012) and colonize the intestinal mucosa by attachment to epithelial cells mediated by adhesins secreted by this pathogen (Farfan & Torres, 2012). Production of *stx*; virulence factors by STEC is responsible for HUS. Due to its clinical significance and ability to cause disease, it has been the subject of many investigations. *stx* are classified into two types, *stx*1 and *stx*2, with *stx*1 having 3 subtypes (a, c, and d), and *stx*2 having 7 (a to g) (Scheutz *et al*., 2012). This pathotype can carry a single variant, stx1 or *stx*2, both *stx*1 and *stx*2, or a combination of *stx*2 subtypes (e.g., *stx*2a and *stx*2c). Both *stx*1- and *stx*2-containing STEC can lead to HUS; however, *stx*2 is more often associated with severe disease (Croxen *et al.*, 2013). Both s*tx*1 and *stx*2 are encoded on prophages that are integrated into the chromosome. Shiga toxin-carrying phages can become lytic during bacterial stress, and it is believed that *stx*1/*stx*2 is released from lysed bacterial cells during the lytic cycle of the phage (Berger *et al*., 2019; Herold *et al.*, 2004). Use of antibiotics to treat STEC infections has become contentious due to the stimulation of the lytic cycle and concomitant toxin

release through the bacterial SOS response. Studies have shown that fluoroquinolones increase *stx*2 production in STEC O157:H7 and that sub-inhibitory concentrations of fluoroquinolones and trimethoprim induce the lytic cycle, while other antibiotics such as azithromycin have no effect (McGannon *et al.*, 2010). The genes; *stx* binds to Gb3 on the surface of endothelial cells (Betz *et al*., 2012) and is internalized and trafficked through the retrograde pathway from the Golgi apparatus and endoplasmic reticulum (ER) and eventually to the host cell cytoplasm. The A subunit is an ribonucleic acid (RNA)-glycosidase that removes an adenine from 28 subunit (28S) ribosomal ribonucleic acid (rRNA), thereby inhibiting protein synthesis and causing cell death. Mechanisms of *stx* transport from the intestinal lumen across the epithelium are unknown but it is hypothesized that STEC-induced inflammation can provide the toxin an opportunity to breach the epithelial barrier (Schüller, 2011) or cross the intestinal epithelium through microfold cells (M cells) and survive in macrophages, thereby release *stx* into the bloodstream, where it can target other organs (Etienne-Mesmin *et al*., 2011). A cluster encoding the cytolethal distending toxin (*cdt*ABC) is found in STEC O157:H7 and less frequently in SFO157:NM isolates. Once delivered into the cell, the enzymatically active CdtB is thought to trigger cell arrest by damaging host DNA and may contribute to HUS. Hemolysin (*hly*A) produced by EHEC is a pore-forming toxin that lyses sheep erythrocytes. The role of Ehx (*ehx*) in virulence is unclear but it has been shown to be cytotoxic to endothelial cells and may contribute to the development of HUS but it is inactivated by EspP (Brockmeyer *et al*., 2011). Several auto transporters are also found in STEC but serine protease EspP is the best studied. A multifunctional protease; EspP cleaves human coagulation factor V, pepsin A, complement and EHEC hemolysin, inactivating its hemolytic activity. All EspP are not secreted except EspPα and EspPγ which are active. Strains of STEC O157:H7 that possess EspPα are most commonly associated with severe disease (Brockmeyer *et al*., 2007; Orth *et al*., 2010). Strains of EHEC also possess LEE genes that encode an effector protein called translocated-intimin receptor (TIR) that is secreted by the T3SS and translocated through EspA filaments into host epithelial cells. TIR localizes in the epithelial cell's cytoplasmic membrane to serve

as a receptor for a LEE-encoded bacterial outer membrane adhesin, called intimin produced by all EHEC strains and related A/E pathogens (Stevens & Frankel, 2014).

2.7.1 Diagnosis of Enterohaemorrhagic *Escherichia coli* infections

Stool samples inoculated onto sorbitol-MacConkey (SMAC) plates and incubated at 37^{o}C in ambient air produce clear colonies within 24 hours. These colonies can then be identified by O157:H7 antisera. Enzyme immunoassay (EIA) kits can be used to identify STEC based on detection of either *Stx*1 or *Stx*2 toxin(s) on the putative isolates (Gould, 2012; Gould *et al*., 2009). However, *stx* can be lost from *in vivo* isolates of O26, SFO157:NM, O103, and O145 serotypes (Gould, 2012; Gould *et al*., 2009), necessitating inclusion of a secondary target like intimin (*sfp* gene) to differentiate O157:H7 from SFO157:NM (Bielaszewska *et al*., 2007, 2008). Serotyping methods have been developed to detect STEC using microarrays, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and microbeads (Lin *et al*., 2011; Norman et al., 2012). Quantitative PCR (qPCR) panels have also been developed to look at multiple genes, such as *rfbE* (O157 antigen), *stx*, *eae*, *ehx*, *fliC*, and O-antigen genes to profile for certain STEC isolates (Gonzales *et al*., 2011). These methods are generally not approved for diagnosis from human samples but may be useful for epidemiological and outbreak studies by public health laboratories.

2.8 Zoonotic *Escherichia coli* infections

Escherichia coli, a Gram-negative bacteria is a known gut commensal of majority of warm blooded animals including NHPs (Bailey & Mansfield, 2010; Clayton *et al*., 2014; Lugano *et al*., 2018). This diverse organism not only plays a role in the maintenance of gut health by helping to prevent the establishment of pathogenic bacteria in the gastrointestinal tract (GIT), but can also exist in a number of pathogenic forms that cause diarrheal illness, life threatening intestinal and extraintestinal infections worldwide (Aminshahidi *et al*., 2017; Gomes *et al*., 2016; Vieira *et al*., 2016).

Strains of *E. coli* that produce cytotoxic necrotizing factors (CNFs) are associated with intestinal and extra-intestinal infections in both humans and animals (Kaper *et al*., 2004). There are three types of CNFs; CNF1, CNF2 and CNF3 with a mechanism of action that involve activation of Rho GTPases, a family of molecular switches with multiple cellular functions, resulting in reorganization of the actin cytoskeleton. Some virulence factors including CNF1, α-haemolysin and P fimbriae are located in the same pathogenicity island. Strains of *E. coli* producing CNF1 are mostly incriminated to cause urinary tract infections in humans. These strains have been isolated from healthy and diseased animal species including weaned pigs and dogs with diarrhoea; cats and dogs with urinary tract infections; ferrets with diarrhoea and extra-intestinal infections; and birds and mink with suspected colibacillosis and coli-septicaemia (Martin *et al*., 2009). Domestic ruminants particularly are the main asymptomatic carriers of *E. coli* O157: H7 with virulence factors like *stx* and *eae* making them potential human pathogens but these bovine pathogens possess fewer virulence factors than human counterparts. However, bovine strains are capable of tolerating adverse conditions than those from human. STEC also colonize gut of sheep and goats among other small stocks. Sporadic isolation of EHEC indicates transmission from humans to wildlife through the environment. For example, there are reports of EHEC infection in deers and gulls (Ferens & Hovde, 2011). EPEC has been isolated as a sole opportunistic pathogen from infant macaques with acquired immunodeficiency syndrome (AIDS) and in co-infection with one or several other enteroparasites. EPEC infections are most common among neonate and infant macaques just like in humans where infants below two years are most susceptible (Mansfield *et al*., 2001).

Five *E. coli* pathotypes; EPEC, ETEC, EAEC, EHEC and EIEC have been isolated from NHPs but there is no established association with diarrhoea (Clayton *et al*., 2014; Kaloppaswamy *et al.*, 2014). It is certainly plausible that these pathotypes could play a role in the intermittent diarrhea observed in primate population. It is likely that EPEC exhibits different effects on different animals, with some animals acting as asymptomatic carriers and others susceptible to diarrhea (Bailey & Mansfield, 2010). Haemorrhagic

diarrhea has been observed in colonies of marmosets. Both EIEC and EHEC have been isolated from cymologus macaques and rhesus macaques implying that they are susceptible to these pathogens (Clayton *et al*., 2014). Pathognomic attachment and effacement lesions have been demonstrated in *Macaca radiata* that were experimentally infected with EHEC O157:H7 (Kang *et al*., 2001). The role of pathogenic *E. coli* in producing gastrointestinal disease in NHPs has not been fully established and the limited available studies have only reported the infection in marmosets, macaques, gorillas, De brazzas, tamarins, lemurs, white spider monkeys among others but not in baboons (Clayton *et al.*, 2014; Kang *et al*., 2001; Kolappaswamy *et al.*, 2014). However, their presence in these NHPs constitute a potential source of DEC that can be transmitted to humans causing a major public health problem.

2.9 Antimicrobial resistance in *Escherichia coli*

Despite being a gut microbiota, *E. coli* is also an essential indicator for dissemination of antimicrobial resistance since it is equally exposed to antibiotics used for treatment of other infections (Stedt *et al.*, 2014). Presence of *E. coli* in livestock that are closely in contact with humans plus their interactions with the wild animal populations including NHPs through shared habitats increases the risk of spreading antimicrobial resistance to a vast range of susceptible hosts even when wildlife has not been exposed to antimicrobial therapies (Guenther *et al*., 2011; Stedt *et al*., 2014; Wallensten *et al*., 2011). In addition, forest fragmentation has increased the interactions between humans and other animals that shed *E. coli* from their GITs and can acquire the bacterium from any of the available hosts whenever they come into contact with their feces (Goldberg *et al*., 2008; Rwego *et al*., 2008). Both bacterial pathogens and commensals in the infected sites of animals including humans are exposed to same groups of antimicrobial agents during chemotherapy (Tadesse *et al.*, 2017). Antimicrobial agents act via different mechanisms like inhibiting synthesis of bacterial cell wall, proteins and nucleic acids synthesis thereby exerting their bacteriocidal or bacteriostatic effects (Kapoor *et al*., 2017). For instance, β-lactam antibiotics that consist of penicillin, cephalosporins, monobactams and carbapenems inhibit bacterial cell wall biosynthesis (Bush & Bradford, 2016). The antibiotic pressure

on the target bacterial pathogens and normal microbial flora including *E. coli* results in development of resistance against these antimicrobial agents and high risk of subsequent transfer (Barlow, 2009). Resistance against β-lactam antibiotics may arise through mutations of target penicillin binding proteins (PBPs), alteration of cell porins to prevent drug from accessing the target site, active efflux of the drug via energy-dependent pumps and hydrolysis of β-lactam ring via the activity of β-lactamases (Zervosen *et al*., 2012)). However, the most common mechanism of antibiotics resistance employed by clinically important Gram-negative bacteria is hydrolysis of β-lactam antimicrobial agents by β-lactamases (Bush & Jacoby, 2010). Most genera of Gram negative bacteria are observed to possess naturally occurring chromosomally mediated β-lactamase that appears to have evolved from PBP due to their sequence homology (Öztürk *et al*., 2015). ESBLs are plasmid-borne enzymes with the ability to hydrolyze oxyimino-cephalosporins and monobactams but not cephamycins and carbapenems (Bradford, 2001). However, ESBLs are susceptible to β-lactam inhibitors including clavulanic acid, sulbactam, tazobactam and avibactam (Perez *et al*., 2016). The ESBLs genes, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ belong to Ambler Class A and are commonly found in Gram negative bacteria including *E. coli* (Bonomo, 2017). The first *bla*$_{\text{TEM,}}$ *bla*$_{\text{TEM-1}}$ was found in *E. coli* isolated from a patient called Temoneira from Athens, Greece and has spread to other bacteria species via plasmids and transposons (Bradford, 2001). There are currently more than 200 *bla*$_{\text{TEM}}$-type β-lactamases and are responsible for approximately 90% ampicillin resistance observed in *E. coli* (ur Rahman *et al*., 2018). The first *bla*$_{\text{SHV}}$ was discovered in 1970 from *E. coli* and denoted as sulfhydryl variable (Liakopoulos *et al*., 2016). Most Gram negative bacteria including clinical *E. coli* isolates harbour *bla*$_{\text{SHV}}$ β-lactamases are encoded in self-transmissible plasmids (ur Rahman *et al.*, 2018). Substitution of amino acids has given rise to 189 *bla*$_{\text{SHV}}$ allelic variants with varying ability to hydrolyze third generation cephalosporins, monobactams and carbapenems (Liakopoulos *et al*., 2016). Over the past two decades, *E. coli* carrying *bla*$_{\text{CTX-M}}$ type ESBL with ability to hydrolyze cefotaxime has been increasingly isolated from nosocomial and community acquired infections (Smet *et al.*, 2010). In addition, *E. coli* strains isolated from healthy humans, livestock, companion animals, food products and sewage have been shown to harbor *bla*$_{\text{CTX-M}}$

ESBLs (Franz *et al.*, 2015; Smet *et al.*, 2010). It has been established that $bla_{\text{CTX-M}}$ gene is the most promiscuous and predominant ESBL (ur Rahman *et al.*, 2018). In most cases, ESBLs are carried on plasmids and other mobile genetic elements that possess genes that encode for resistance against other antimicrobial agents like aminoglycosides and sulphonamides (Bush & Jacoby, 2010) making them highly transmissible. Presence of antimicrobial resistance determinants in *E. coli* from olive baboons and/or other NHPs could be a major threat to its rapid spread to humans that get into contact due to settlement and other anthropogenic activities (Goldberg *et al*., 2008; Lugano *et al*., 2018) with subsequent development and/or dissemination of community acquired antimicrobial resistance. Carriage of ESBLs by both pathogenic and non-pathogenic *E. coli* is well documented in humans (Bryce *et al*., 2016; Franiczek *et al.*, 2012), but in NHPs which are phylogenetically related to humans, the data is limited. These groups of animals are known to share same habitat and thus the possibility for a bidirectional transmission of antibiotic resistant bacteria including *E. coli.* This can result in emergence or re-emergence of infections that are difficult to treat due to limited and/or inaccessible treatment options especially in low income countries leading to high morbidity and mortality (Ghaderpour *et al.*, 2015; Planta, 2007). There is limited data on antimicrobial susceptibility patterns of *E. coli* among NHPs and this study provides a window of determining the burden of the problem.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

This study was conducted at the Institute of Primate Research (IPR) that is located within the expansive Oloolua forest near Karen, 20 Km from Nairobi city in Kenya. It ethically utilizes NHPs including olive baboons for biomedical research as guided by national and international guidelines (National Research Council, 2011). This institution is a World Health Organization (WHO) collaborating centre with its laboratory animal facility accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mpala ranch is a 48,000 acre property ate the heart of Laikipia County northwest of Mt. Kenya. This is a home to more than 25 wild mammalian species including baboons that closely interact with pastoral communities and their livestock making it an ideal model for studying various aspects of transmission of zoonoses (Mpala Research Center, 2020).

3.2 Study design

This was a cross-sectional study that involved screening two groups of olive baboons. One group comprised of captive baboons housed within IPR's animal enclosures. Captive population was made up of 62 olive baboons housed in a group cage where they interact socially. The other group designated as wild comprised an equal number of free-ranging baboons within Mpala ranch located in Laikipia County.

3.3 Sample size calculation

Sample size was calculated using the formulae shown below (Hajian-Tilaki, 2011; Kadam & Bhalerao, 2010):

$$n = \frac{2(Z_{\alpha}+Z_{1-\beta})^2 \acute{p}(1-\acute{p})}{(p1-p2)^2}$$

Where:

n = Minimum samples required during the study

Z_{α}= 1.96

$Z_{1-\beta}$=0.842

P1= 0.6 prevalence of DEC in rhesus and cymolgus macaques (Kolappaswamy *et al*., 2014)

P2 = 0.32 prevalence of diarrhea in captive marmosets (Carvalho *et al*., 2003)

$$\acute{p} = \frac{p1+p2}{2}$$

The sample size (N) required was:

$$N= 2\frac{(1.96+0.842)^2\ 0.46x0.54}{(0.60-0.32)^2}$$

= 50 animals per group

3.4 Study Animals

This study was conducted using a total of 124 healthy adult baboons divided into two equal groups of 62; captive and wild olive baboons. The first group consisted of captive baboons that were housed in standard animal facility within IPR where the animals are provided with adequate enrichment in social group enclosures that minimizes stress. All NHPs are fed on commercial monkey chow (Unga Farm Care Limited, Nairobi Kenya) supplemented with fruits, vegetables and water *ad libitum*. The second group comprised of free ranging wild baboon troops found within Mpalla ranch conservancy located at Laikipia County, Kenya.

3.5 Sample collection and processing

3.5.1 Stool sample collection

Samples of both captive and wild baboons were collected from freshly voided feces using sterile cotton tipped applicator swab moistened in normal saline, with care to collect from

the top of the sample to avoid ground contamination. These stool samples were clearly labeled with the appropriate animal identity (e.g. Pan 001). All fecal swabs were aseptically inoculated into Stuart transport media (Oxoid, Basingstoke UK), placed on ice, transported to the laboratory and subsequently processed.

3.5.2 Bacterial isolation and identification

Each stool sample was inoculated into MacConkey broth (Oxoid, Basingstoke UK) and incubated for six hours. A loopful was then sub-cultured onto Xylose Lysine Deoxycholate (XLD) (Oxoid, Basingstoke UK) and MacConkey agar (Oxoid, Basingstoke UK) plates that were incubated at 35^{o}C in ambient air for 24 hours. Sorbitol MacConkey agar (Oxoid, Basingstoke UK) plates were included in order to effectively isolate *E. coli* O157:H7. Inoculated plates were examined for lactose fermentation in order to classify the isolates as lactose fermenters and/or non-lactose fermenters. All Lactose fermenters were subjected to Gram staining in order to study their morphology and Gram reaction. Colonies that appeared as Gram negative rods were sub-cultured onto MacConkey agar (Oxoid, Basingstoke UK) and incubated at 37^{o}C for 24 hours in order to obtain pure isolates that were used for biochemical tests to identify them. Colonies of pure lactose fermenters isolates that were less than 24 hours old were suspended in 5 ml sterile physiological saline to form a turbidity of 0.5 McFarland. This suspension was aseptically inoculated into twenty biochemical test tubes of analytical profile index 20 Enterobacteriaceae (API 20E, BioMeriux® SA, Marcy l'Etoile, France) and incubated at 36^{o}C for 24 hours according to manufacturer's instructions while a colony of the same isolate was tested for production of oxidase enzyme (Oxoid, Basingstoke UK) to complete the API 20E profile. After 24 hours inoculated test strips were examined and the results recorded on the coupons supplied with the kits. Isolated Gram negative rods were identified using APIWEB standalone software (Ali *et al*., 2012). Isolated *E. coli* were suspended in Tryptone soy (Oxoid, Basingstoke UK) broth mixed with glycerol and stored at -20^{o}C awaiting molecular characterization and subsequent antimicrobial sensitivity testing.

3.5.3 DNA isolation

In order to prepare *E. coli* template DNA, the isolates were inoculated into Tryptone soy broth and incubated at 37^{o}C for 24 hours. After 24 hours, 2 ml of Tryptone soy broth isolate cultures were centrifuged at 10,000 rpm. The supernatant was discarded into a container containing 5000 ppm chlorine disinfectant leaving a pellet rich in *E. coli* isolate at the bottom of the tube that was re-suspended in 0.5 ml of sterile nuclease free water then boiled at 100^{o}C for 10 minutes in a heat block followed by spinning the lysate at 10000 rpm for 5 minutes to obtain template DNA in the supernatant which was carefully transferred to another tube (Bölin *et al*., 2006; Xia *et al.*, 2010) and stored at -20^{o}C.

3.5.4 Characterization of *E. coli* pathotypes

In order to characterize *E. coli* pathotypes, isolated DNA of the presumptive *E. coli* isolates were amplified in 0.2ml PCR reaction tubes containing10 µl 5xPCR buffers, 0.2mM dNTPs mixture, 2.5 U *Taq* DNA polymerase, 0.15 µmol of each primer and1µltemplate DNA to make a total reaction volume of 20µl. DNA templates from known *E. coli* pathotypes were used as positive controls during the assay. Primers listed in Table 3-1 were used in this study to target *elt* and *est* for ETEC, *eae* and *bfp*A for EPEC, CVD432 for EAEC, *eae, stx*1&2 (VTcom) for EHEC/STEC, *ipa*H for EIEC as a multiplex assay. Thermocycling condition for all reactions involved initial denaturation step of 2 minutes at 95^{o}C followed by 30 cycles of 15 seconds denaturation at 95^{o}C, 8seconds annealing at 52^{o}C and 10seconds extension at 72^{o}C together with a final extension for 2 minutes at 72^{o}C (Tobias & Vutukuru, 2012). Amplified PCR products were subjected to electrophoresis on 1.5% agarose gel then visualized using ultra-violet (UV) transilluminator documentation system (UVP Bio-Doc It™ Imaging System, Upland, CA, USA). This involved mixing 10 µl of PCR products including the positive and negative controls with the loading dye, loading carefully into the wells of pre-cast 1.5% agarose gel placed in an electrophoresis tank. A 100 bp molecular marker (Thermo Scientific, Lithuania, UK) was put in wells on both ends of the agarose gels in order to determine the size of the amplicons. The electrophoresis tank was connected to the power supply

and allowed to run for one hour. The agarose gel was then removed from the tank carefully and placed on a UV transilluminator for visualization. Amplicons sizes were then determined and interpreted against reference primers (Table 3.1) with the aid of a 100 bp molecular marker (Thermo Scientific, Lithuania, UK).

Table 3.1: Primers used for identification of *E. coli* Pathotypes

Oligonucleotide sequence (5'-3')	Target gene	Product size (bp)	Reference
ETEC ACGGCGTTACTATCCTCTC **TGGTCTCGGTCAGATATGTG**	*Elt*	273	(Tobias & Vutukuru, 2012)
ETEC TCTTTCCCCTCTTTTAGTCAG **ACAGGCAGGATTACAACAAAG**	*est*A1	166	(Rodas *et al*., 2009)
ETEC TTCACCTTTCCCTCAGGATG **CTATTCATGCTTTCAGGACCA**	*est*A2-4	120	(Rodas *et al*., 2009)
EPEC GGAAGTCAAATTCATGGGGGTAT **GGAATCAGACGCAGACTGGTAGT**	*bfpA*	300	(Vidal, *et al*, 2004)
EPEC TCAATGCAGTTCCGTTATCAGTT **GTAAAGTCCGTTACCCCAACCTG**	*eae*	482	(Vidal, *et al*, 2004)
EHEC GAGCGAAATAATTTATATGTG **TGATGATGGCAATTCAGTAT**	*stx*1+ *stx*2	518	(Tobias & Vutukuru, 2012)
EIEC GTTCCTTGACCGCCTTTCCGATACCGTC **GCCGGTCAGCCACCCTCTGAGAGTAC**	*ipa*H	600	(Aranda *et al*., 2007)
DAEC CTGGCGAAAGACTGTATCAT **AAATGTATAGAAATCCGCTGTT**	pCVD432	630	(Rodas *et al.*, 2009)

3.5.5 Antimicrobial susceptibility testing

All *E. coli* isolates from the two groups of baboons were screened for antimicrobial resistance using Kirby-Bauer agar disk diffusion method on Mueller-Hinton agar (Becton Dickinson and Co. Sparks NV, USA) according to Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2015). All antimicrobial disks were sourced from Becton Dickinson and Co. Disks containing the following commonly used antimicrobial agents were used: Ampicillin (AM) 10 µg, Chloramphenicol (C) 30 µg, Tetracycline (TE) 30 µg, Gentamycin (GM) 10 µg, Streptomycin (S) 10 µg, Trimethoprim/Sulphomethoxazole (SXT) 25 µg, Norfloxacin (NOR) 10 µg, Ciprofloxacin (CIP) 5 µg, Cefaclor (CEC) 30 µg, Ceftriazone (CRO) 30 µg, Cefotaxime (CTX) 30 µg, Cefuroxime (CXM) 30 µg, Cefepime (FEP) 30 µg, and Amoxicillin/Clavulanic acid (AMC) 20/10 µg. *E. coli* ATCC 25922 was used as the quality reference strain. Diameters of zones of inhibition were measured in millimeters and interpreted against CLSI standards (CLSI, 2015).

3.5.6 Detection of ESBLs' phenotypes

Phenotypic screening of ESBLs was performed by double disk synergy test followed by genotypic detection using polymerase chain reaction (PCR). Four disks of antimicrobial agents; CAZ 30 µg, FEP 30 µg, CTX 30 µg and CRO 30 µg (Liofilchem s.r.l Zona Industriale, 64026, Roseto degli Abruzzi (Te) Italy) were placed onto isolated *E. coli* inoculum on Mueller-Hinton agar (Becton Dickinson and Co. Sparks NV, USA) to surround a centrally positioned AMC 30 µg (Liofilchem s.r.l. Zona Industriale, 64026, Roseto degli Abruzzi (Te) Italy) 30 mm apart from each cephalosporin. These plates were incubated for 24 hours then examined for enhanced zone(s) of inhibition between AMC 30 µg in the middle and any of the four cephalosporins. Enhanced zone of inhibition indicated synergistic activity and production of ESBL. *E. coli* isolates that were positive for double disk synergy test were further tested against Ceftazidime/Clavulanic acid (CAL) 40 µg and CAZ 30 µg alone for confirmation. The strains that had a zone of

inhibition around CAL acid which exceeded that of CAZ alone by 5 mm were considered to produce ESBL enzymes (Ahmed *et al*., 2013).

3.5.7 Detection of ESBL genotypes

Genotypic detection of ESBL was conducted by PCR that targeted three putative genes; *bla*$_{TEM}$ (β-lactamase Temoniera), *bla*$_{CTX-M}$ (β-lactamase cefotaximases) and *bla*$_{SHV}$ (β-lactamase sulfhydryl variable) (Table 3.2). PCR for detecting each gene was conducted using 5 µl of *E. coli* DNA template (section 3.5.3), 1 µl of each 10 pM primer (Integrated DNA Technologies Inc., Illinois, USA), 12.5 µl DreamTaq PCR mastermix 2x (Thermo Scientific, Lithuania, UK) then topped up with nuclease free water to obtain a final reaction volume of 25 µl (Lim *et al.*, 2009; Rezai *et al.*, 2015). All the reactions were performed in a thermocycler (SimpliAmp™ Thermocycler, AppliedBiosystems, ThermoFisher Scientific, Singapore) pre-set at conditions described by Oliver *et al* (2002) and Pagani *et al* (2003). Detection of *bla*$_{TEM}$ and *bla*$_{SHV}$ required initial denaturation for 5 min at 96^{o}C followed by 35 cycles of 1 min denaturation at 96^{o}C, 1 min annealing at 58^{o}C and 1 min extension at 72^{o}C with a final extension for 10 min at 72^{o}C.Thermocycling conditions for detection of *bla*$_{CTX-M}$ consisted of initial denaturation for 7 min at 94^{o}C, 35 cycles 50 sec denaturation at 94^{o}C, annealing for 40 sec at 50^{o}C and extension for 1 min at 72^{o}C with a final extension for 5 min at 72^{o}C. *Klebsiella pneumoniae* ATCC 700603 was used as a positive control while *E.* coli ATCC 25922 was the non-ESBL producing control in the entire experiment. Amplified PCR products were subjected to electrophoresis on 1.5% agarose gel then visualized using transilluminator (UVP Bio-Doc It™ Imaging System, Upland, CA, USA). Amplicons sizes were then determined and interpreted against reference primers (Table 3.2) with the aid of a 100 bp molecular marker (Thermo Scientific, Lithuania, UK).

Table 3.2: Primers used for detection of ESBL resistant genes

Oligonucleotide sequence (5'-3')	Target gene	Product Size (bp)	Reference
ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	*bla*$_{TEM}$	867	(Oliver *et al.*, 2002)
GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	*bla*$_{SHV}$	867	(Oliver *et al*., 2002)
ATGTGCAGYACCAGTAARGT TGGGTRAARTARGTSACCAGA	*bla*$_{CTX-M}$	593	(Pagani *et al.*, 2003)

3.6 Ethical clearance

This study was reviewed and approved by the IPR's Scientific and Ethical Review committee (ISERC). This involved rigorous scrutiny of all protocols to ensure that animals are handled in compliance with recommended animal care and use practices. Use of stool samples in studies that utilize laboratory animals is considered non-invasive and therefore advocated whenever possible since it does not subject the subjects to unnecessary stress. This project was assigned an approval reference ISERC (ISERC/12/15) after completion of the review process.

CHAPTER FOUR

RESULTS

4.1 Identification of *Escherichia coli* pathotypes

A total of 124 *E. coli* were isolated from both captive (n=62) and wild (n=62) baboons. Four pathotypes; ETEC, EPEC, EHEC and EIEC were prevalent in the captive 15 (24.2%) population of baboons (Table 4.1). On the other hand three pathotypes; EPEC, EIEC and ETEC were isolated from 18 (29.0%) wild baboons (Table 4.1). Carriage of ETEC was higher among captive 9 (14.5%) baboons compared to the wild. Wild baboons were observed to harbour more EPEC 14 (22.6%) than the captive animals ($p<0.05$; Appendix IV). However, EAEC and DAEC were not isolated from stool samples from both groups of animals.

Table 4.1: *E. coli* pathotypes isolated from captive and wild olive baboons

Pathotype	Number (%) of pathotypes isolated	
	Captive (n*=62)	Wild (n*=62)
Enterotoxigenic *E. coli*	9 (14.5%)	1 (1.6%)
Enteropathogenic *E. coli*	3 (4.5%)	14 (22.6%)
Enterohaemorrhagic *E. coli*	2 (3.2%)	0
Enteroinvasive *E. coli*	1 (1.6%)	3 (4.8%)
Enteroaggregative *E. coli*	0	0
Diffuse enteroaggregative *E. coli*	0	0

*n=number of olive baboons that were sampled from each group

Isolates of *E. coli* are characterized by fermentation of lactose on XLD agar plates cultures. Colonies of lactose fermenter appear yellow in colour on XLD agar plates (Fig. 4.1).

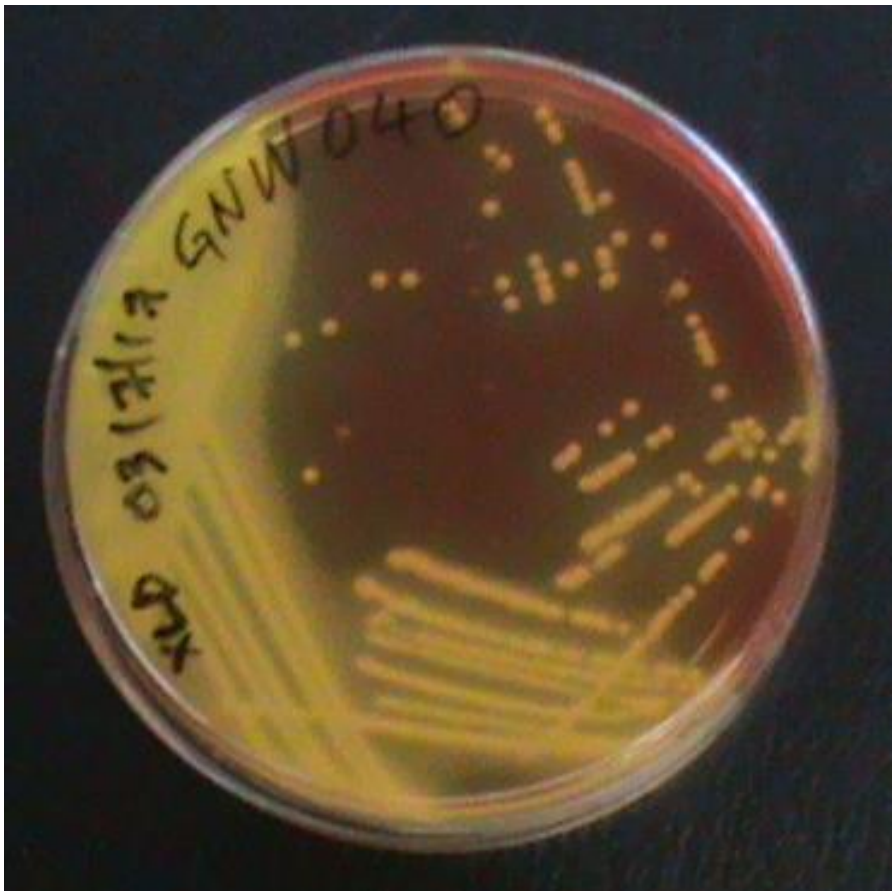

Figure 4.1: Xylose lysine deoxycholate agar plate showing colonies of lactose fermenters.

Yellow colonies indicate lactose fermenters a characteristic associated with *E. coli.* This was used for presumptive identification of isolates from baboon fecal samples. All yellow colonies were presumptively considered as *E. coli* and subjected to subsequent identification tests (Gram staining and biochemical tests).

Further identification of *E. coli* isolates was accomplished using Gram stain. Isolated *E. coli* appeared as pink rod-shaped organisms (Fig. 4.2) when stained with Gram stain.

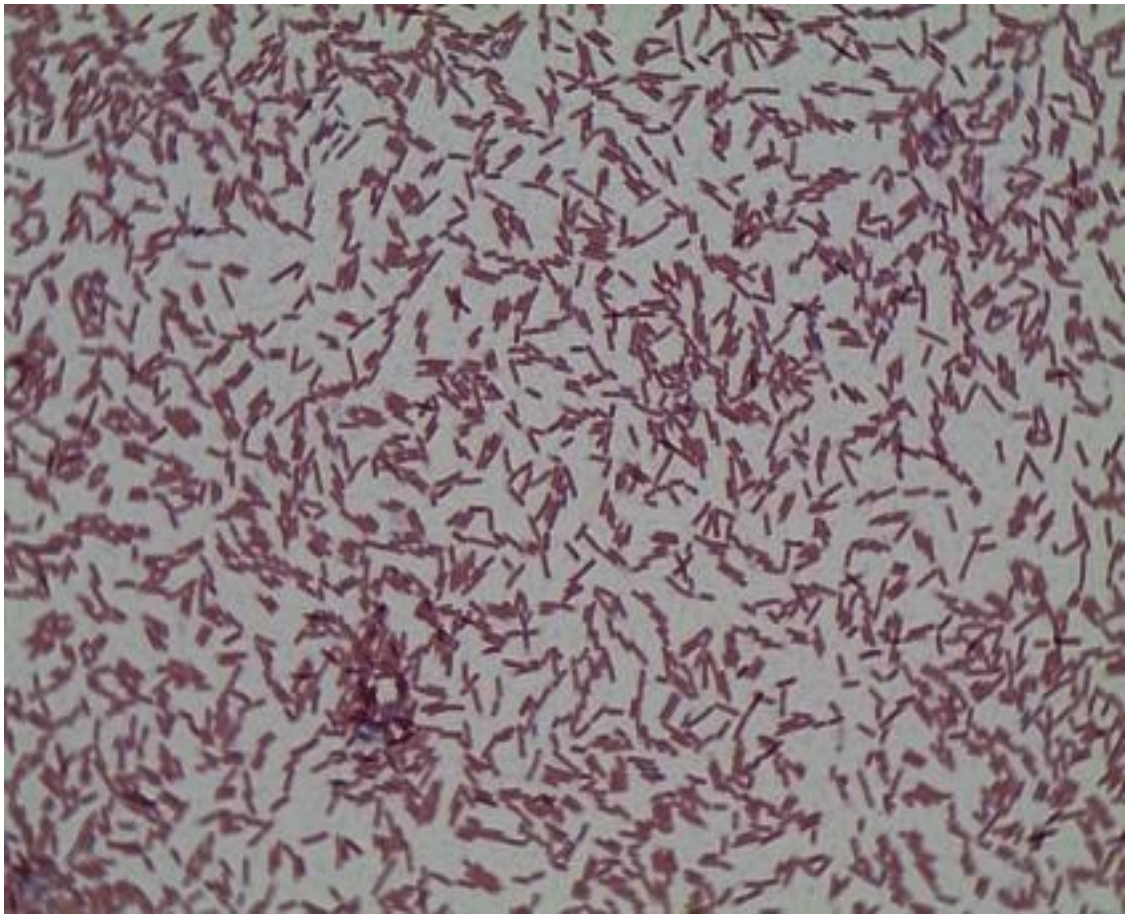

Figure 4.2: Microscopy of a Gram stained slide.

A Gram stained slide showing Gram negative rods; a characteristic of *E. coli* (×1000 magnification) isolated from baboon fecal samples.

Confirmation of *E. coli* isolates was accomplished subjecting them to 20 biochemical tests that demonstrated production of lactose permease depicted by positive O-nitrophenyl-p-galactopyranoside (ONPG), decarboxylation of lysine (LDC) and ornithine (ODC) but not arginine (ADH) by E. coli (Appendix III). Strains of *E. coli* do not utilize citrate (CIT), produce Hydrogen sulfide (H_2S) or urease enzyme (URE). It deaminates (TDA) and decomposes (IND) tryptophan but does not ferment sugars with production of acetoin

(VP). It does not liquify gelatin (GEL) but ferments all sugars in the API 20E strip except inositol (INO) and amygdalin (AMY). All isolates with these biochemical tests characteristics were identified as *E. coli* (Fig. 4.3).

Figure 4.3: API 20E results positive for *E. coli* isolates.

4.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was undertaken on all *E. coli isolates* by disk diffusion method where fourteen antibiotics were used. The isolates from both groups of animals were prevalently resistant to Ampicillin (32.3%) captive and (35.5%) wild baboons. Isolates from feces of captive baboons showed higher prevalence of antimicrobial resistance against SXT (37.1%), AMC (25.8%) and S (11.7%) than those from their wild counterparts ($p<0.05$; Appendix V). All *E. coli* isolates from both groups of animals were susceptible to CIP whereas they were resistant to the other antimicrobial agents used in this study (Fig. 4.4).

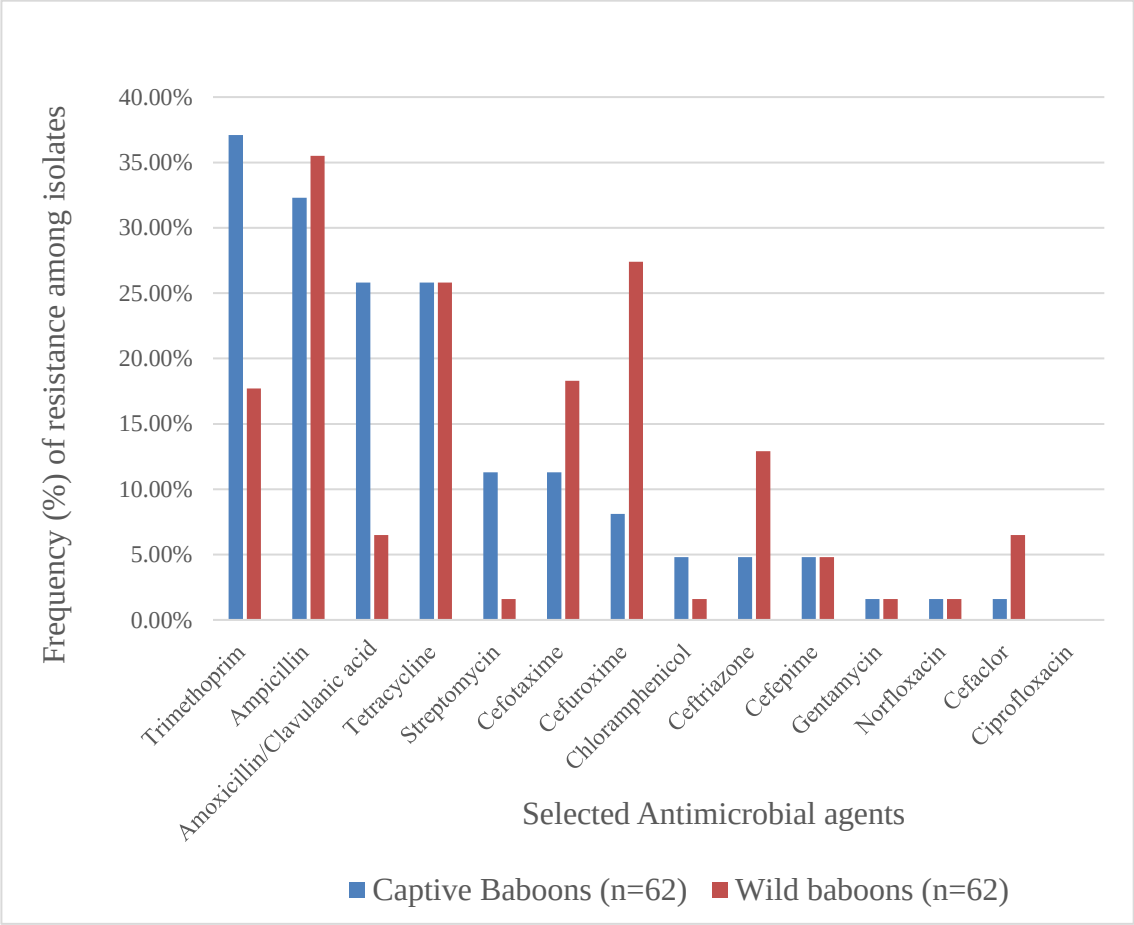

Figure 4.4: Antimicrobial susceptibility patterns of *E. coli* isolates from captive and wild baboons

4.3 Detection of Genes Encoding extended spectrum β-lactamases

A total of 124 *E.* coli isolates, 62 each from captive and wild baboons were tested for three ESBL genes. The prevalence of the genes was marginally higher in *E. coli* isolated from wild 11 (17.7%) than the captive 9 (14.5%) baboons ($p>0.05$; Appendix VI). However, the isolates harbouring ESBL genes; $bla_{\text{CTX-M}}$ 5 (8.1%) and bla_{TEM} 3 (4.8%) were more dominant among captive baboons population (Fig. 4.6). On the other hand presence of bla_{SHV} gene was higher in *E. coli* isolated from wild baboons 7 (11.3%) compared to the captive 1 (1.6%) population (Fig. 4.6).

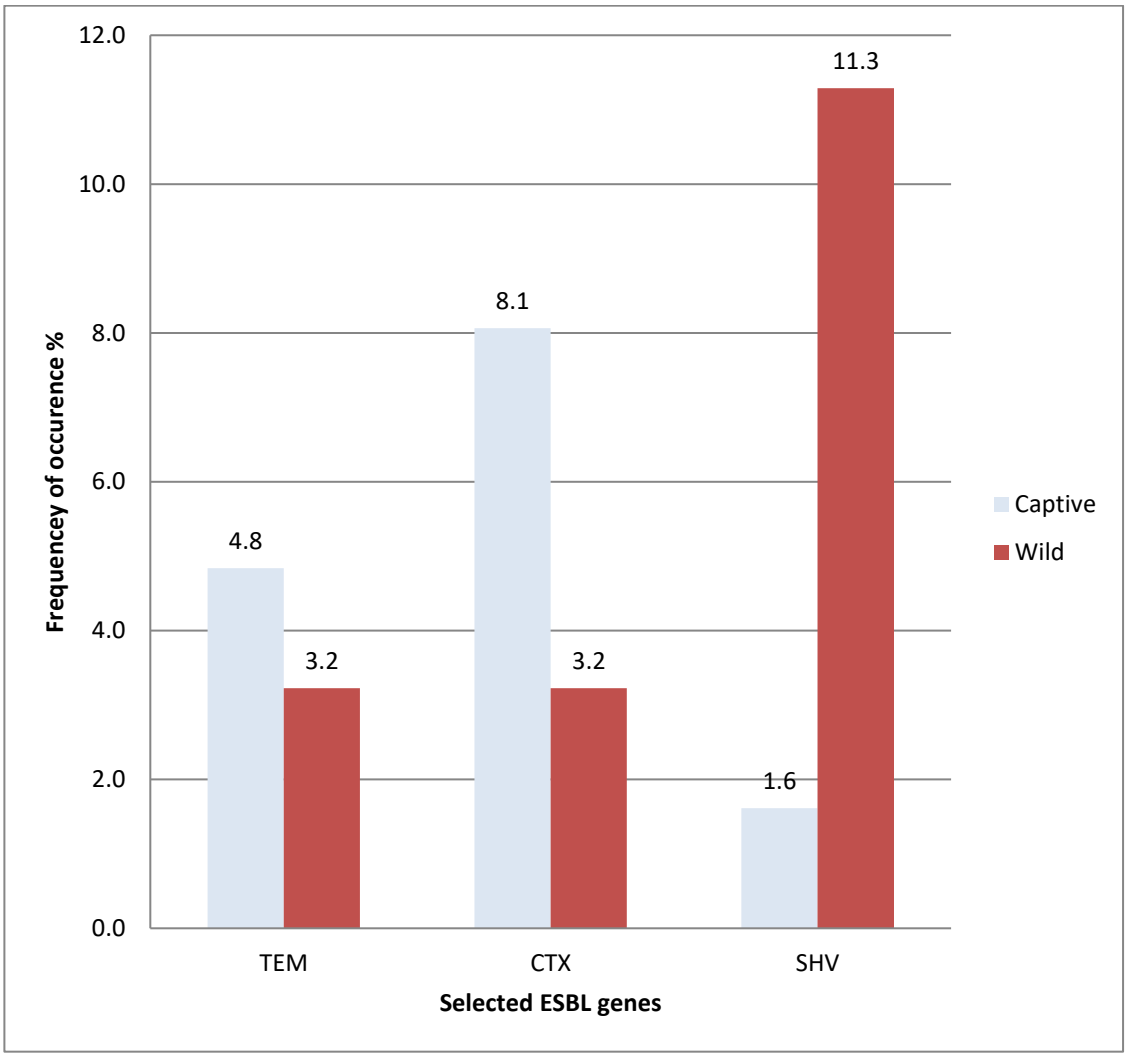

Figure 4.5: ESBLs genotypes in *E. coli* isolates from captive and wild baboons

Phenotypic determination of ESBL genes was accomplished by double disk synergy test (Fig. 4.5). The illustrated 'ghost' zones produced between third generation cephalosporins and amoxycillin/clavulanic acid, a feature consistent with ESBL production by bacteria. Isolated *E. coli* showing enhanced zones of inhibition between amoxycillin/clavulanic acid (AMC) (at the centre) and cefepime (FEP), ceftriazone (CRO) and ceftazidime (CAZ).

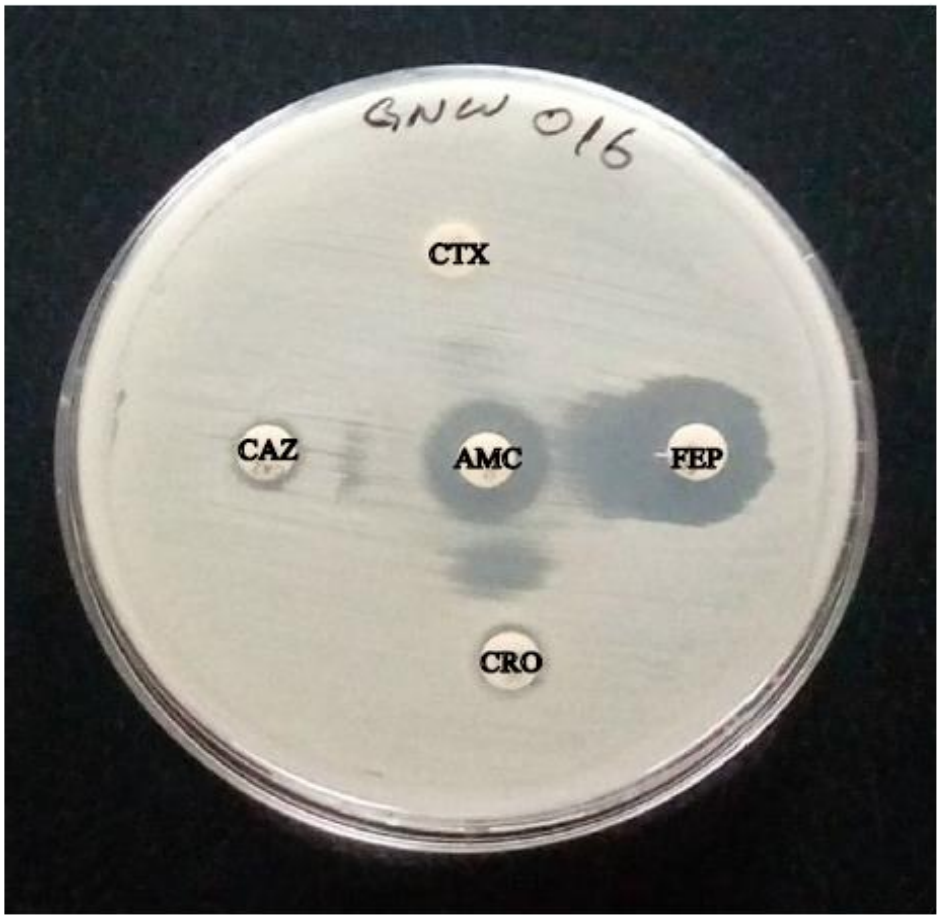

Figure 4.6: Demonstration of ESBLs Phenotypic screening of *E. coli* isolates

Visualization of amplicons sizes corresponding to gene that code for *bla*$_{CTX-M}$ on agarose gel after electrophoresis as illustrated in Fig. 4.7.

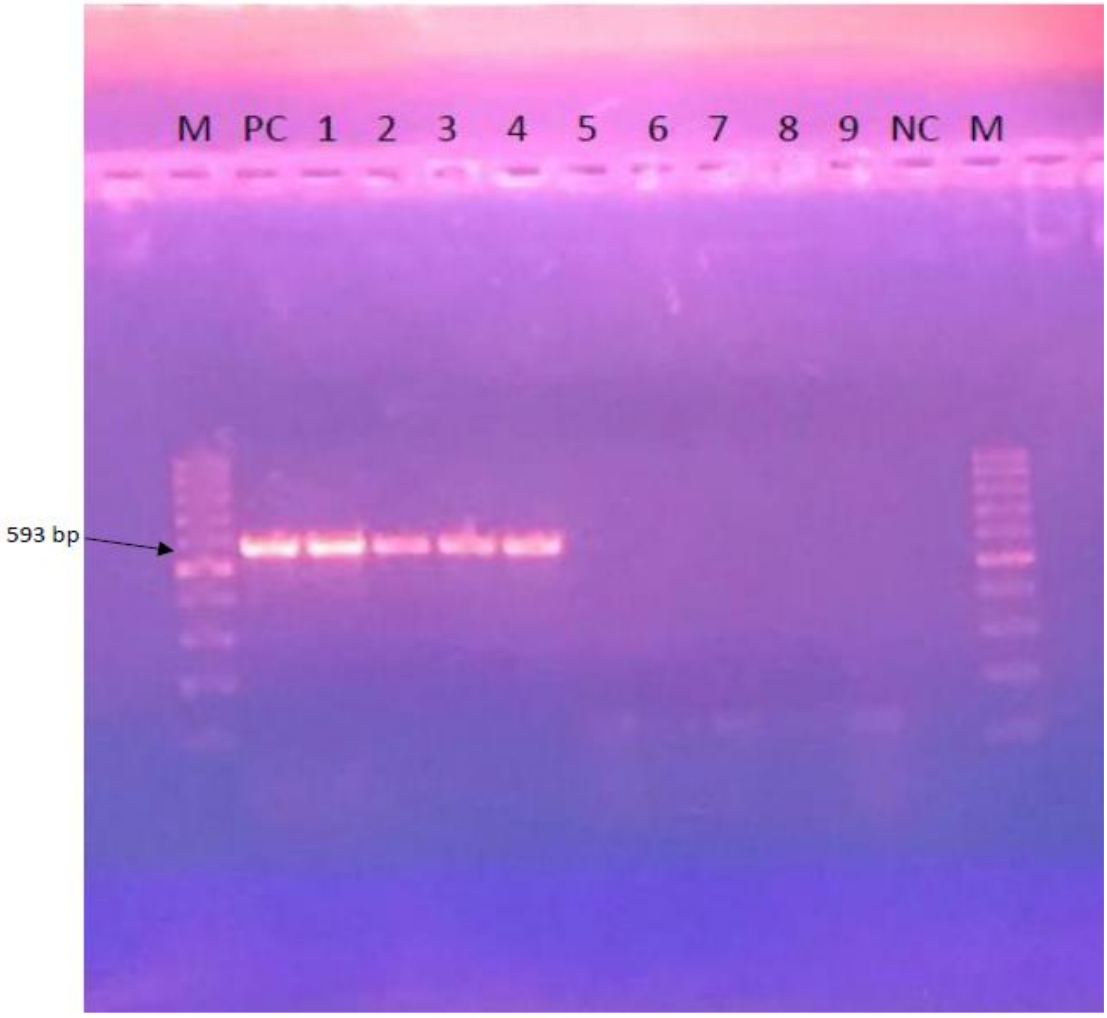

Figure 4.7: Agarose gel electrophoresis showing *bla*$_{CTX-M}$

M-100bp molecular marker, PC-positive control (*K. pneumoniae* ATCC 700603), 1-4-samples with positive amplification, 5-9-samples with negative amplification and NC-Negative control (*E. coli* ATCC 29522).

Demonstration of amplicons sizes corresponding to gene that code for *bla*$_{TEM}$ generated from agarose gel electrophoresis of PCR products as demonstrated in Fig. 4.8.

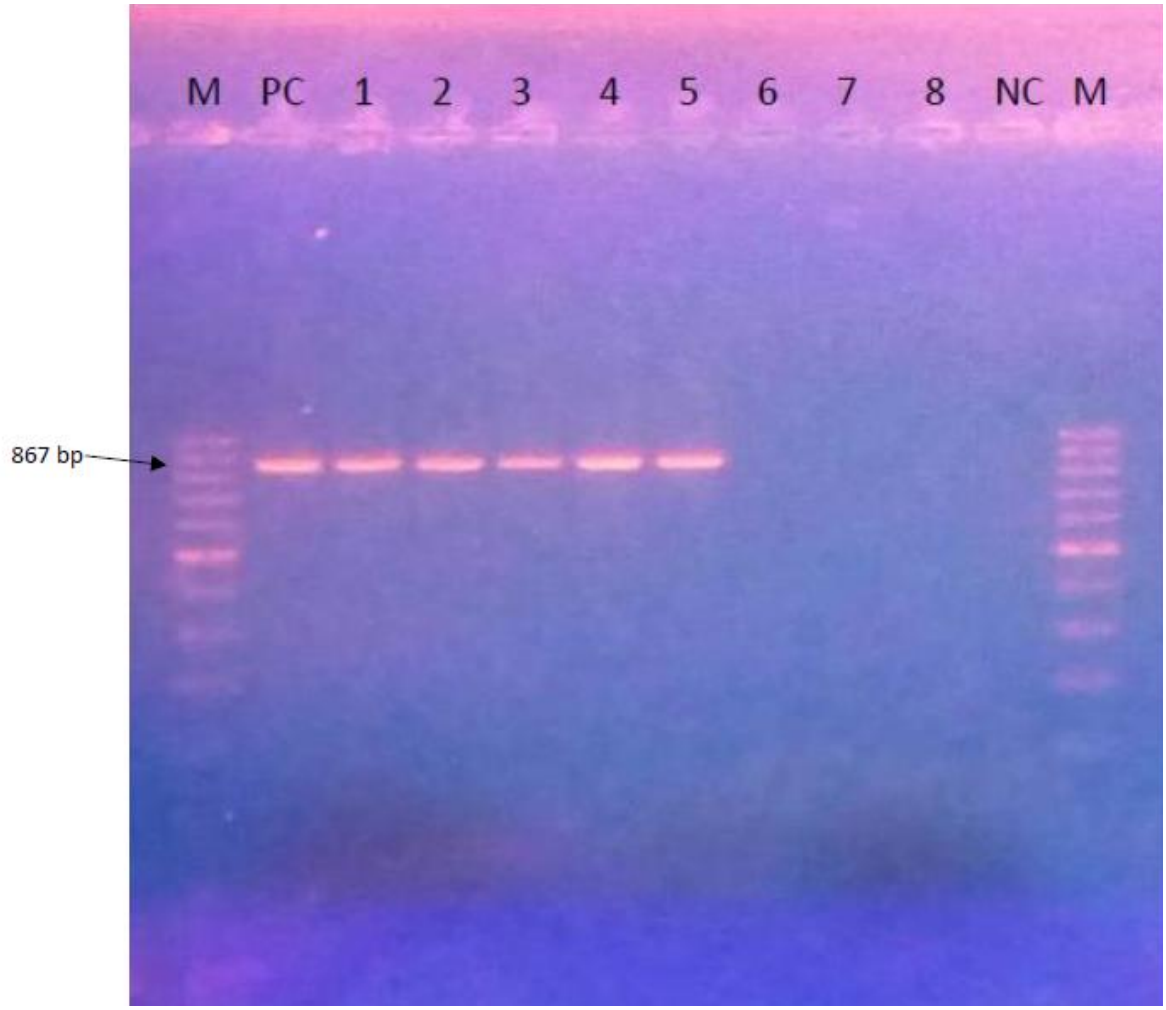

Figure 4.8: Agarose gel electrophoresis showing *bla*$_{TEM}$ amplification

M-100bp molecular marker, PC-positive control (*K. pneumoniae* ATCC 700603), 1-5-samples with positive amplification, 6-8-samples with negative amplification and NC-Negative control (*E. coli* ATCC 25922).

Agarose electrophoresis demonstrating PCR products corresponding to genes that code for *bla*$_{SHV}$ detected from isolated *E. coli* is illustrated in Fig 4.9.

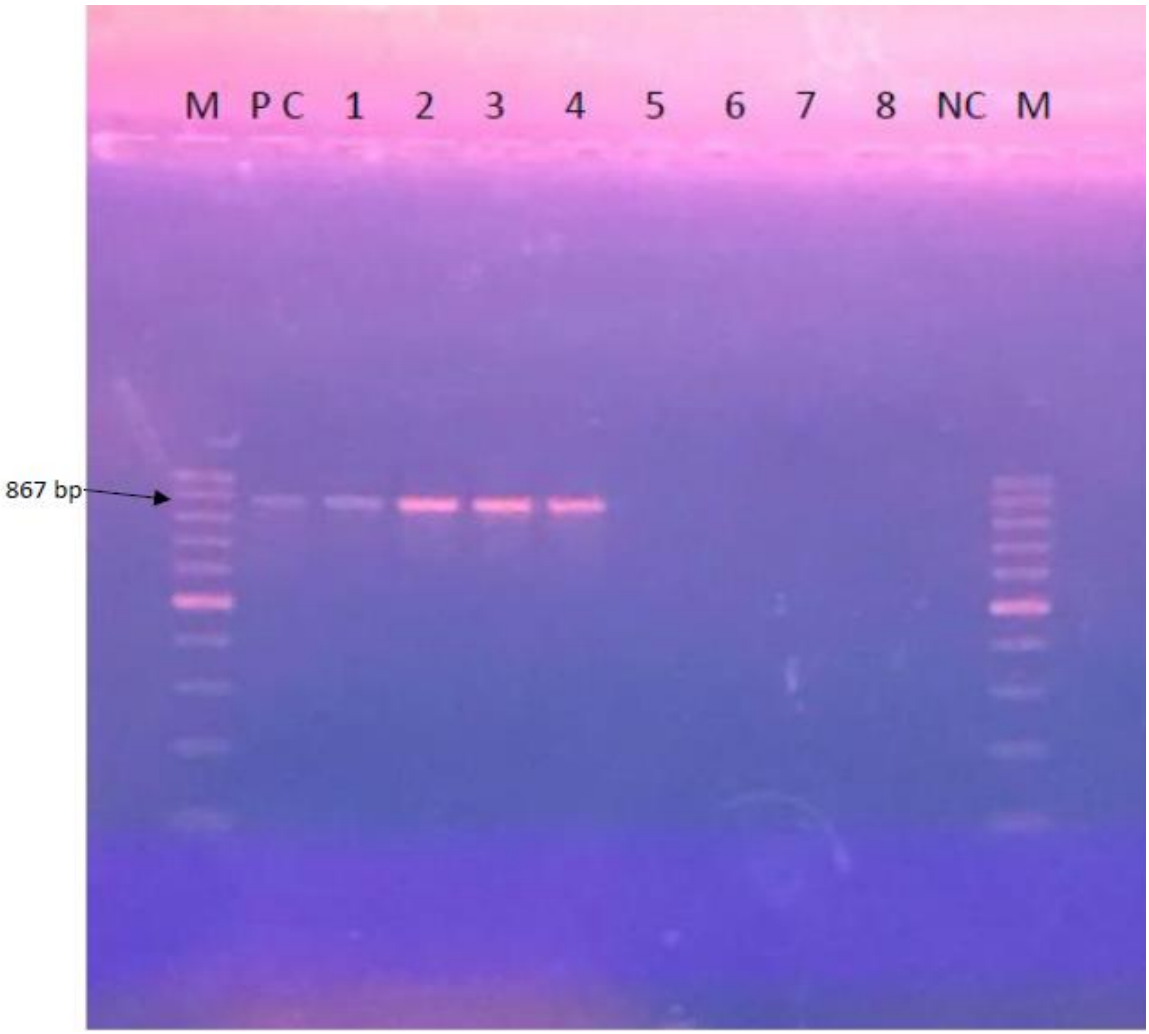

Figure 4.9: Agarose gel electrophoresis showing *bla*$_{SHV}$ amplification

M-100bp molecular marker, PC-positive control (*K. pneumoniae* ATCC 700603), 1-4-samples with positive amplification, 5-8-samples with negative amplification and NC-Negative control (*E. coli* ATCC 25922).

CHAPTER FIVE

DISCUSSION

5.1 Pathotypes of *Escherichia coli* isolates from captive and wild olive baboons

This study established presence of four *E. coli* pathotypes; ETEC, EPEC, EIEC and EHEC in faecal samples of the two groups of baboons. The observed difference in the prevalence where three pathotypes namely; ETEC, EPEC and EIEC were detected in 29.0% of wild olive baboons fecal samples whereas four pathotypes; ETEC, EPEC, EIEC and EHEC were isolated from 24.2% of the captive group could be attributed to variations in the environmental settings where these populations were sampled from. A study undertaken by Kaloppaswamy *et al* (2014) established that fecal samples from NHPs including De Brazzas, spider monkeys, white-faced sakis, lemurs and tamarins were prevalently contaminated with either of the three pathotypes; EPEC, EHEC and EIEC detected in this study. However, in another study aimed at determining gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock, it was established that interaction of these animal species increased zoonotic transmission (Rwego *et al*., 2008). The captive baboons are socially housed within IPR with stringent husbandry conditions where they routinely closely interact with animal health care personnel and thus there is a high possibility of transmission of these pathotypes during cleaning, feeding and other experimental procedures (Muriithi *et al*, 2015). Routine introduction of environment enrichment material could also be an important source of infection among the captive baboons. On the other hand, wild baboons consisted of free-ranging troops that are frequently in close contact with humans, livestock and other feral animals within the region that increase their risk of transmission including *E. coli* pathotypes (Goldberg *et al*., 2008). The four pathotypes; ETEC, EPEC, EIEC and EHEC that were detected in fecal samples from the study baboons have also been demonstrated in fecal samples collected from different healthy NHPs in a zoo setting (Clayton *et al.*, 2014). This implies that NHPs can be an important source of DEC infection to humans who come into contact with their fecal materials by virtue of occupation or anthropogenic activities. However, fatal

outbreaks of diarrhoea caused by EIEC have been reported in rhesus macaques and another one by EHEC in cynomolgus macaques (Kaloppaswamy *et al*., 2014) but not in baboons. Presence of these microbes in baboons and other NHPs in captivity and wild is not uncommon since generally it has been proven difficult to eliminate most infectious pathogens from these laboratory animals even when different approaches that work in other species have been employed (Bailey & Mansfield 2010). This is partly attributed to the fact that unlike most laboratory animals, NHPs require to be socially housed in highly enriched enclosures similar to their natural habitat for both welfare and breeding purposes (National Research Council, 2011) and this promotes introduction and transmission of pathogens. Neither EAEC nor DAEC were detected in all the baboon faecal samples analyzed, an these two have hardly been previously found in NHP gut *E. coli* isolates except the autotransporter enterotoxin gene; *pet* of EAEC that is responsible for secretory diarrhoea (Clayton et al., 2014; Navarro-Garcia & Elias, 2011).

However, the outbreaks of diarrhea associated with EPEC producing characteristic A/E lesions has been reported in 47% of New world monkeys (Carvalho *et al*., 2003). In addition, opportunistic EPEC infection has been observed among simian immunodeficiency virus infected rhesus macaques (Mansfield *et al*., 2001). Atypical EPEC O98 have been isolated from 2.6% golden snub-nosed monkeys in China that had diarrhea (Qi *et al*., 2017). These pathotypes have been demonstrated to cause fatal outbreaks in humans (Clements *et al*., 2012; Croxen *et al*., 2013; Vieira *et al*., 2016) thus presenting them as important zoonoses. Infections with DEC pathotypes is mostly associated with childhood diarrhoea where moderate to severe cases are reported globally (Thakur *et al*., 2018). The importance of DEC in development of paediatric diarrhoea cannot be overstated in developing countries (Saka *et al*., 2019) including Kenya where cases are increasingly being reported (Shah *et al*., 2016). Animals including NHPs have been reported as reservoirs for aEPEC (Delahoy *et al*., 2018). None of the animals from both groups used in this study showed signs of diarrhea suggesting carriage of these pathotypes by olive baboons which means that human interaction with their fecal matter could result in zoonotic transmission of DEC.

5.2 Antimicrobial susceptibility profiles of *Escherichia coli* isolated from captive and wild olive baboons.

This study demonstrated that *E. coli* isolates from both captive and wild olive baboons were resistant to all fourteen antimicrobial agents that we used except CIP. High prevalence of antimicrobial resistance against AM (32.3%; 35.5%), TE (25.8%; 25.8%) and STX (37.1%; 17.7%) observed in *E. coli* isolates from both captive and wild baboons could be attributed to direct or indirect exposure to antimicrobial agents (Aminov, 2009; Davies & Davies, 2010). Higher prevalence of *E. coli* resistant to SXT (37.1%), AMC (25.8%) and S (11.3%) among captive baboons was observed compared to those from the wild group ($p<0.05$) could be as a result of antibiotic pressure due to chemotherapy during routine husbandry procedures (Norris *et al*., 2019; Prestinaci *et al*., 2015). The observed higher prevalence of resistance against four cephalosporins including CXM (27.4%), CTX (18.3%), CRO (12.9%) and CEC (6.5%) by *E. coli* isolates from wild baboons compared to the captive could be attributed to exposure of bacteria to anthropogenically contaminants that provoke them to develop mechanisms of resisting activity to antimicrobial agents like non-specific efflux (Davies & Davies, 2010). Proximity of wild baboons as they raid crops (Hill, 2000; Wallace & Hill, 2012) and also due to human animal conflicts in search for arable lands, poaching, logging among others are vehicles for transmitting antibiotic resistant microbes to the feral settings (Goldberg *et al*., 2008). These results are consistent with the findings of a previous study that was conducted on animals not previously exposed to antimicrobial chemotherapy (Muriithi *et al.*, 2015). A study conducted on free-ranging yellow baboon troops from Amboseli National Park in Kenya revealed high prevalence of antimicrobial resistance against Tetracycline (94.1%), Kanamycin (70.6%), Ampicillin (47.1%) and Cephalothin (17.6%) but third generation cephalosporins and ESBLs were not included (Rolland *et al.*, 1985). This high prevalence of antibiotic resistance observed in baboons from Amboseli National Park was attributed to foraging on food wastes and other refuse that could be implicated as the source of antibiotic resistant non-pathogenic bacteria including *E. coli* (Rolland *et al.*, 1985). This also explains our findings since the wild baboons sampled in this study closely interact

with humans and different types of wastes within Mpala ranch. This could be reinforced by the fact that vehicles of transmission of *E. coli* comprise of human and NHPs fecal material in areas where there is close contact during human activities in the animals infested areas and contaminated water bodies (Goldberg *et al*., 2008). Transmission of bacteria including antimicrobial resistant *E. coli*, between different susceptible hosts like humans, livestock, NHPs and other wild animals due to forest fragmentation (Chapman *et al.*, 2005) increases risk of infections whose interventions are scarcely available or lacking. Infections by antibiotics resistant organisms are associated with poor clinical outcomes including prolonged hospitalization, economic pressure and increased mortality (Thaden *et al*., 2017). The interspecies transmission is accelerated by the ecologic overlap created by the fragments and anthropogenic activities in the affected regions with these resistant microbes spreading to the community settings (Rwego *et al*, 2008).

Public health problem of antimicrobial resistance is emphasized by the high prevalence against SXT (80.6-95.2%), AM (77.4-95.2%), TE (57.1-81.0%), C (14.3-35.7%), GM (6.4%) and CIP (3.2%) that has been reported among pathogenic *E. coli* isolated from humans from different parts of Kenya (Sang *et al.,* 2012). Close interaction between animals including baboons and humans in conflict increases the risk of transmission of microbes like *E. coli* that have been subjected to constant antimicrobial pressure through livestock farming, poor human waste disposal and polluted environment (Martinez, 2009). In addition to being part of the essential gut microbial flora, *E. coli* is responsible for paediatric septicaemia and community-acquired sepsis in sub-Saharan Africa (Williams *et al*., 2018) adding to the burden of morbidity which when coupled with antimicrobial resistance as revealed in this study deteriorates to a grave public health problem.

5.3 Selected β-lactamase genes detected in *Escherichia coli* isolates from captive and wild olive baboons.

This study established that *E. coli* isolated from both groups of baboons harboured all the three ESBL resistance genes under investigation namely $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. However, the observed difference in prevalence where the three ESBLs were detected in

17.7% of wild baboons compared to 14.5% of the captive group could be ascribed to ubiquity of resistance genes that can be easily acquired from contaminated soils (Prestinaci *et al*., 2015) in feral free-ranging environment in Mpala ranch compared to exposure to resistant bacteria in potentially contaminated enrichment materials in animal enclosures (Mansfield *et al*., 2001). Higher prevalence of *bla*$_{SHV}$ that was detected in 11.3% of wild baboons and 1.6% of the captive animals could be as a result of presence of antibiotics resistance genes in environmental bacteria with determinants that are readily transmissible by horizontal gene transfer to resident *E, coli* in these hosts (Martínez *et al*., 2007; Martinez, 2009). Detection of 8.1% and 4.8% *E. coli* harbouring *bla*$_{CTX-M}$ and *bla*$_{TEM}$ respectively in captive baboons compared to 3.2% of each gene among the wild counterparts could be attributed to antibiotic selective pressure during routine chemotherapy and acquisition of resistance determinants (Martinez, 2009). Antimicrobial resistance that was observed among the wild population could be attributed to habitat contamination by human or domestic animal waste containing antibiotic residues or gut microbial flora harboring resistance genes (Doi *et al*., 2010). Presence of ESBL genes detected in *E. coli* isolates from the two groups of animals could be attributed to the conjugational transfer of plasmid-mediated ESBLs occurs efficiently in the intestinal tract, where enteric rods, often act as a reservoir of self-transmissible resistance markers that can be exchanged between species of the *Enterobacteriaceae* family (Franiczek *et al*., 2012). This phenomenon of gene transfer is on the premise that ESBLs can be borne on plasmids and mobile genetic elements makes them highly transmissible horizontally resulting into 'super' pathogens (Doi *et al*., 2010). This has posed AMR as a significant threat to the prevention and treatment of bacterial infections (Bernabé *et al.*, 2017; Tadesse *et al*., 2017). Previous studies have reported that commensals from healthy individuals including *E. coli* are reservoirs for highly transmissible antibiotics' resistance genetic material that can be readily acquired by pathogens (Okeke *et al.*, 2007). Genes that encode for ESBLs that include *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ have been detected in *E. coli* isolated from clinical specimens in different parts of the world with reported upsurge of MDR that complicate morbidity caused by this bacterium (Lim *et al*., 2009). Carriage of ESBL genes has been detected in *E. coli* isolates from human clinical specimens at both hospital and

community settings (Lim *et al*., 2009; Bajaj *et al*., 2016). Successful management of infections caused by such resistant strains requires an understanding of the diversity of β-lactamases, their unambiguous detection, and molecular mechanisms underlying their expression and spread with regard to the most relevant information about individual bacterial species (Bajaj *et al*., 2016).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study established the following conclusions:

Four pathotypes; ETEC, EPEC, EIEC and EHEC were identified in the two groups of baboons. Three pathotypes; ETEC, EPEC and EIEC were detected in fecal samples collected from 18 (29.0%) of wild olive baboons. Four pathotypes; ETEC, EPEC, EIEC and EHEC were isolated from fecal samples collected from 15 (24.2%) of the captive olive baboons. The most prevalent pathotype among the captive baboons was ETEC 9 (14.5%) whereas prevalence of EPEC 14 (22.6%) was highest in the wild population.

Multidrug resistant *E. coli* isolates were detected from both captive and wild olive baboons. However, *E. coli* isolated from captive baboons displayed high resistance to SXT (37.1%), AMC (25.8%) and S (11.7%) compared to those from the wild.

All the three ESBL genes under investigation in this study including bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ were detected in *E. coli* isolated from both captive and wild olive baboons. This therefore renders them a serious potential threat in the transmission of these genes among human, domestic and wild animal communities surrounding their habitats.

6.2 Recommendations

- There is need to sensitize laboratory animal handlers of the potential of zoonotic transmission of DEC and ESBLs from baboons.
- Awareness campaigns aimed at creating awareness of health risks associated with human-animal interactions should be strengthened.
- Further investigation of variants of the three ESBL genes under investigation in this study.

- Molecular characterization of additional genes encoding AMR should be undertaken on *E. coli* and other enteric pathogens of human health concern.

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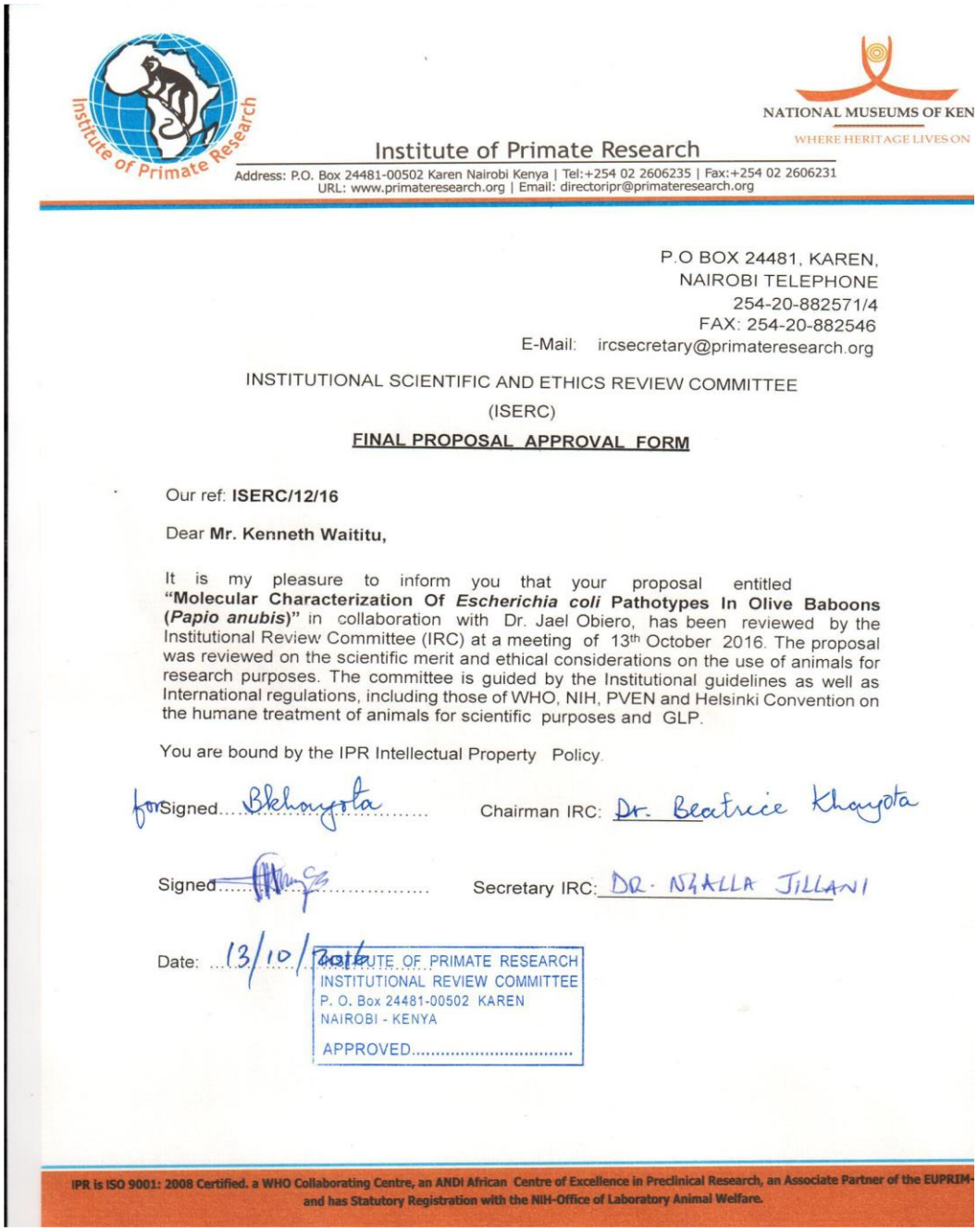
APPENDICES

Appendix I: Letter of Scientific and Ethical approval



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INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE
(ISERC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **ISERC/12/16**

Dear **Mr. Kenneth Waititu,**

It is my pleasure to inform you that your proposal entitled **"Molecular Characterization Of *Escherichia coli* Pathotypes In Olive Baboons (*Papio anubis*)"** in collaboration with Dr. Jael Obiero, has been reviewed by the Institutional Review Committee (IRC) at a meeting of 13th October 2016. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

You are bound by the IPR Intellectual Property Policy.

for Signed..... Chairman IRC: Dr. Beatrice Khayota

Signed..... Secretary IRC: DR. NJALLA JILLANI

Date: 13/10/2016

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED.....

IPR is ISO 9001: 2008 Certified. a WHO Collaborating Centre, an ANDI African Centre of Excellence in Preclinical Research, an Associate Partner of the EUPRIM- and has Statutory Registration with the NIH-Office of Laboratory Animal Welfare.

Appendix II: Abstract of the published article

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Antimicrobial Susceptibility Patterns of *Escherichia coli* Isolated from Olive Baboon (*Papio anubis*) Gut

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Abstract **Background**: Antimicrobial resistance is widely acknowledged as a global health problem that has resulted in devastating emerging and re-emerging conditions which are difficult to manage due to limited or unavailable intervention options. It is deepened by the fact that genes encoding for antimicrobial resistance can be transferred horizontally by mobile genetic elements. *Escherichia coli* is primarily a gut microbial flora in warm-blooded animals including non-human primates that can acquire any of these gene elements from other resistant bacterial strains resulting in their transmission between humans and animals. This study aimed to determine antimicrobial susceptibility of *E. coli* against commonly used agents as well as production of extended spectrum β-lactamases. **Methods**: *E. coli* was isolated from stool samples that were collected from sixty-two captive and sixty-two wild baboons using culture-based methods. The isolates were subjected to fourteen antimicrobial agents followed by characterization of three putative resistance genes; bla_{CTX-M}, bla_{TEM} and bla_{SHV} using polymerase chain reaction. **Results**: *E. coli* isolates from both groups of animals were resistant to all antimicrobial agents except Ciprofloxacin. Prevalence of Ampicillin resistance was high in *E. coli* isolated from both captive (32.3%) and wild (35.5%) baboons. There was higher prevalence of ESBLs in *E. coli* isolated from wild (17.7%) than captive (14.5%) baboons. **Conclusion**: As reservoirs of ESBL producing *E. coli* type, baboons could play a potential role in antibiotic resistant plasmids transmission to the environment and other animals including humans.

Keywords: *Escherichia coli, ESBL, antimicrobial, wild, captive*

Cite This Article: Waititu Kenneth Kariuki, Kimang'a Andrew Nyerere, Kariuki Samuel, and Obiero Jael Apondi, "Antimicrobial Susceptibility Patterns of *Escherichia coli* Isolated from Olive Baboon (*Papio anubis*) Gut." *American Journal of Infectious Diseases and Microbiology*, vol. 6, no. 2 (2018): 38-45. doi: 10.12691/ajidm-6-2-1.

1. Introduction

Globally, there is a growing concern over antimicrobial

pathogenic bacteria in the gastrointestinal tract (GIT), but can also exist in a number of pathogenic forms that cause diarrheal illness, life threatening intestinal and extraintestinal infections worldwide [9]. Since this bacterium is equally exposed to antibiotics used for treatment of other

Appendix III: API 20E test description

Tests	Substrates	Quantity	Enzymes/ Reactions	Results	
ONPG	ortho-nitrophenyl–D-galactopyranoside (ONPG) isopropyl-thiogalactopyranoside (IPTG)	0.2 mg	β-galactosidase	Negative	Positive
				Colorless	Yellow (1)
ADH	L-Arginine	2.0 mg	Arginine Dihydrolase	Yellow	Red /Orange (2)
LDC	L-Lysine	2.0 mg	Lysine Decarboxylase	Yellow	Red/Orange (2)
ODC	L-Ornithine	2.0 mg	Ornithine Decarboxylase	Yellow	Red /Orange (2)
CIT	Tri-Sodium citrate	0.8 mg	Citrate Utilization	Pale Green / Yellow	Blue-Green/Blue (3
H_2S	Sodium thiosulfate	80.0µg	H_2S production	Colorless / Greyish	Black Deposit / Thin Line
URE	Urea	0.8 mg	Urease	Yellow	Red/Orange (2)
TDA	L-Tryptophane	0.4 mg	Tryptophane deaminase	Add one drop of TDA ($Fe(Cl_2)$ and read immediately	
				Yellow	brown-red
IND	L-Tryptophane	0.2 mg	Indole production	Add James reagent (1 drop) / Immediately	

				Colourless, Yellow/pale green	Pink
VP	Creatine Sodium pyruvate	0.9 mg 2.0 mg	Acetoin production	VP1 (1 drop) + VP2 (1 drop) / 10 min	
				Colorless/pale pink	pink / red (5)
GEL	Kohn's charcoal (gelatin-bovine origin)	0.6 mg	Gelatinase	no diffusion of black pigment	diffusion of black pigment
GLU	Glucose	2.0 mg	Fermentation / oxidation (4)	blue / blue-green	Yellow / greyish yellow
MAN	Mannitol	2.0 mg			
INO	Inositol	2.0 mg			
SOR	Sorbitol	2.0 mg			
RHA	Rhamnose	2.0 mg			
SAC	Sucrose	2.0 mg			
MEL	Melibiose	2.0 mg			
AMY	Amygdalin	0.57mg			
ARA	Arabinose	2.0 mg			

1) A very pale yellow should also be considered positive.
2) An orange color after 36-48 hours incubation must be considered negative.
3) Reading made in the cupule (aerobic).
4) Fermentation begins in the lower portion of the tubes, oxidation begins in the cupules.
5) A slightly pink color after 10 minutes should be considered negative.

Appendix IV: Statistical comparison of *E. coli* pathotypes isolated from captive and wild baboons

Pathotype	Number (%) of pathotype isolated	
	Captive (n*=62)	Wild (n*=62)
Enterotoxigenic *E. coli*	9 (14.5%)	1 (1.6%)
Enteropathogenic *E. coli*	3 (4.5%)	14 (22.6%)
Enterohaemorrhagic *E. coli*	2 (3.2%)	0
Enteroinvasive *E. coli*	1 (1.6%)	3 (4.8%)
Enteroaggregative *E. coli*	0	0
Diffuse enteroaggregative *E. coli*	0	0

*n=number of olive baboons that were sampled from each group

X^2 test, df 3=8.16906

X^2 test, (CI=95%); df 3= 7.81

$P<0.05$

Appendix V: Statistical comparison Antimicrobial susceptibility pattern of *E. coli* from captive and wild baboons

Antimicrobial agent	Number (%) of resistant isolates	
	Captive (n*=62)	Wild (n*=62)
Trimethoprim	23 (37.1%)	11 (17.7%)
Ampicillin	20 (32.3%)	22 (35.5%)
Amoxicillin/Clavulanic acid	16 (25.8%)	4 (6.5%)
Tetracycline	16 (25.8%)	16 (25.8%)
Streptomycin	7 (11.3%)	1 (1.6%)
Cefotaxime	7 (11.3%)	11 (18.3%)
Cefuroxime	5 (8.1%)	17 (27.4%)
Chloramphenicol	3 (4.8%)	1 (1.6%)
Ceftriazone	3 (4.8%)	8 (12.9%)
Cefepime	3 (4.8%)	3 (4.8%)
Gentamycin	1 (1.6%)	1 (1.6%)
Norfloxacin	1 (1.6%)	1 (1.6%)
Cefaclor	1 (1.6%)	4 (6.5%)
Ciprofloxacin	0 (0.0%)	0 (0.0%)

*n=number of olive baboon that were sampled. $P<0.05$

X^2 test, df 13=42.42

X^2 test, (CI=95%); df 13= 22.36

$P<0.05$

Appendix VI: Statistical comparison Selected ESBL genes detected in *E. coli* from captive and wild baboons

ESBL gene	Number (%) of selected resistance genes	
	Captive (n*=62)	Wild (n*=62)
TEM	3 (4.8%)	2 (3.2%)
CTX	5 (8.1%)	2 (3.2%)
SHV	1 (1.6%)	7 (11.3%)

X^2 test, df 2=5.844156

X^2 test, (CI=95%); df 2= 5.991

P>0.05

Appendix VII: Directions to Mpala Ranch from Nanyuki